MARTA FILIPA DE SOUSA RIBEIRO

# IMMUNOMODULATORY EFFECTS INDUCED BY EXTRACELLULAR PRODUCTS OF GLIOBLASTOMA CELL CULTURES

Dissertação de candidatura ao grau de Mestre em Oncologia – especialização em Oncologia Molecular – submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

**Orientador** – Pedro Jorge Fonseca Madureira, PhD
**Categoria** – Investigador auxiliar
**Afiliação** – Instituto de Investigação e Inovação em Saúde da Universidade do Porto.

**Coorientador** – Professora Doutora Berta Martins da Silva
**Categoria** – Professora Associada
**Afiliação** – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

*“Learn from yesterday, live for today, hope for tomorrow. The important thing is never stop questioning.”*

**Albert Einstein**

# AGRADECIMENTOS

Embora uma tese seja, pela sua finalidade académica, um trabalho individual, há um conjunto de pessoas e contributos de natureza diversa que se revelam decisivos para a concretização dos objetivos pretendidos. Por estas razões desejo expressar os meus sinceros agradecimentos a todos aqueles que, em diferentes períodos e diferentes formas me ajudaram nesta árdua caminhada.

Ao Pedro Madureira, que qualidade de orientador me proporcionou a oportunidade de integrar um projeto inovador. Agradeço-te por me deixares fazer parte do teu trabalho, por me mostrares como uma investigação deve ser conduzida e, principalmente, por todo o conhecimento e ideias que me transmitistes que, sem margens de dúvidas, contribuíram largamente para o meu desenvolvimento, não só pessoal, mas principalmente cientifico. Obrigado por me transmitires o teu entusiasmo pela Imunologia, que é fascinante. Agradeço-te a disponibilidade, a simpatia, a compreensão, a boa disposição, a paciência, as criticas, as correções e as sugestões. Obrigado por todo o apoio que me transmitiste nesta etapa fulcral de preparação para a minha futura carreira na investigação científica. Foi um enorme prazer trabalhar contigo.

À Professora Doutora Berta Martins da Silva que na qualidade de Diretora do Mestrado em Oncologia, docente da cadeira de Imunologia e Cancro, responsável pelo laboratório de Imunogenética do ICBAS e, em especial, por ter aceite ser coorientadora deste projeto e por me transmitir o gosto pela Imunologia. Agradeço por me ter acolhido no seu laboratório, por toda a disponibilidade e simpatia e por toda a sabedoria partilhada.

À Sara Santos e à Mafalda Saraiva agradeço pelos conselhos, pelo apoio e principalmente pelos bons momentos. Agradeço-vos o carinho e a boa disposição de todos os dias, a disponibilidade para ajudar no que fosse preciso e as conversas alegres e enriquecedoras. Obrigado por todo o vosso empenho, pela vossa paciência e por tornarem tão mais fácil e mais divertido o nosso trabalho no laboratório. Considero que esta tese também é vossa, pois sem dúvida sem o vosso contributo não teria chegado à “meta”. Sempre, desde o primeiro minuto, que funcionamos como uma equipa e trabalhamos como tal. Muito obrigado!

À Profª Doutora Cármen Jerónimo, e ao Grupo de Epigenética do Cancro do CI-IPO Porto, por terem cedido uma das linhas celulares e pela partilha de conhecimento quanto às culturas de células.

À Doutora Paula Soares, coordenadora do grupo *Cancer signalling and Metabolism* do I3S, e ao Mestre Ricardo Coelho, do grupo *Differentiation & Cancer* do I3S, por terem cedido algumas das linhas tumorais estudadas neste projeto.

A todo o Grupo de Imunobiologia do I3S, por me ter acolhido e ajudado na etapa final deste projeto. Em especial, à Filipa Lemos, por toda a ajuda e paciência, e por todas as horas passadas no citómetro, à Cristiana Nogueira, ao Tiago Rodrigues, à Ana Marques e à Doutora Carla Teixeira por toda a ajuda e por toda a partilha de conhecimentos.

Aos Docentes do 1º ano do Mestrado pelo conhecimento transmitido que enriqueceu a minha formação científica.

Às mestres Sandra Brás, Bárbara Leal, Cláudia Carvalho e Daniela Bolexa pela disponibilidade, prontidão e ajuda. Sem dúvida que a vossa ajuda foi fundamental. Agradeço também a Encarnação por estar sempre pronta ajudar e pela simpatia.

A todos os amigos e família que me incentivaram e me deram ânimo para alcançar os meus objetivos.

À Natália, pela amizade e pelos conselhos. Obrigado por tudo, apesar da distância, estás sempre presente.

Ao Gerson, por tudo! Por simplesmente fazeres parte da minha vida e me contagiares com a tua força e a tua vontade de vencer, por maiores que sejam as adversidades. És sem dúvida um exemplo a seguir. Obrigado pela paciência, pela confiança, pela valorização do meu trabalho, por me ajudares em tudo que podes, por me acalmares nas situações mais difíceis e festejares comigo as minhas vitórias como se fossem as tuas. Obrigado por tudo!

Por último, mas muito importante, aos meus Pais por todo o amor e apoio incondicional, pelo incentivo, pela disponibilidade e pela confiança que depositaram em mim durante todo o meu percurso académico. As palavras não são suficientes para vos agradecer por tudo que fizeram e fazem por mim. Obrigado por serem um exemplo de humildade e determinação e obrigado por toda a força que me dão para nunca desistir. Agradeço pela educação que me deram e por tudo aquilo que me ensinaram, por todas as condições que sempre me deram para o meu crescimento, pelas chamadas de atenção e por exigirem o melhor de mim. Se hoje posso dizer que tenho orgulho no que me tornei devo-o inteiramente a vocês. Obrigado por serem os Melhores Pais do Mundo! Este trabalho é dedicado a vocês.

# RESUMO

O cancro é uma das patologias mais agressivas e letais, permanecendo um desafio para investigadores e médicos, mesmo após décadas de investigação e investimento nesta área. A iniciação e progressão tumoral é processo complexo que depende de diversas interações entre o tumor e os tecidos circundantes. A capacidade dos tumores iludir a destruição pelo sistema imune, tornou-se um emergente *Hallmark* do cancro.

O glioblastoma multiforme (astrocitoma grau IV) é o tipo mais comum e agressivo de tumor maligno cerebral. Pacientes com este tumor apresentam um prognóstico desfavorável e um tempo de sobrevida muito baixo. Deste modo, torna-se emergente a criação de novas terapias que superem as atuais limitações do tratamento.

O objetivo principal deste estudo foi avaliar a capacidade imunomodeladora de produtos extracelulares de células de glioblastoma*, in vitro*. Para tal, foi avaliada a capacidade dos produtos extracelulares de culturas de células de glioblastoma recolhidos a diferentes tempos, quer em induzir a produção de IL-10 pelas células T quer em inibir a ativação de células T. Também foi avaliado o efeito dos produtos extracelulares na função e na diferenciação dos macrófagos.

Os resultados mostraram que produtos extracelulares de células de glioblastoma têm uma capacidade imunomodeladora nas células imunitárias do hospedeiro. Mais especificamente, observou-se que estes produtos extracelulares foram capazes de inibir a ativação das células e induzir a produção de IL-10 em culturas de PBMC. Interessantemente, os produtos excretados pelas células de glioblastoma também induzem ativamente a polarização de monócitos em macrófagos M2, um fenótipo associado com uma resposta anti-inflamatória ou imunossupressora. É importante referir que estes efeitos podem ser observados mesmo se os produtos extracelulares forem obtidos a tempo muito precoces de culturas de células de glioblastoma.

Em conclusão, estes resultados permitiram inferir um impacto dos produtos extracelulares de células de glioblastoma na supressão da resposta imune.

## ABSTRACT

Cancer is one of the most aggressive and lethal diseases, remaining a challenge for researchers and physicians despite decades of research and investment in this area. Tumour initiation and progression is a complex process that depends on several interactions between the tumour and surrounding tissues. The ability of tumours to evade its own destruction by the immune system has become an emerging hallmark of cancer.

Glioblastoma multiforme (astrocytoma grade IV) is the most common and aggressive type of malignant brain tumour. Patients with this type of tumours have a poor prognosis and a low median overall survival. Thus, it becomes urgent to develop new therapies that overcome the current limitations of treatment.

The aim of this study was to evaluate, in vitro, the immunomodulatory capacity of extracellular products of glioblastoma cells. To this end, it was evaluated the ability of extracellular products of glioblastoma cell cultures recovered at different time points, either to induce IL-10 production by T-cells or to inhibit T cell activation. It was also evaluated the effect of extracellular products in function and differentiation of macrophages.

The results showed that extracellular products of glioblastoma cells have an immunomodulatory ability on host immune cells. More specifically, we observed that these extracellular products were able to inhibit T cell activation and to inducing IL-10 production by in PBMC cultures. Interestingly, the products excreted by glioblastoma cells also actively induce the polarization of monocytes into M2 macrophages, a phenotype associated with an anti-inflammatory or immune-suppressive response. Importantly, these effects could be observed even if the extracellular products were obtained at very early time points of glioblastoma cell cultures

In conclusion, these results allow to infer an impact of extracellular products of glioblastoma cells in suppression of the immune response.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATION

**α:** alpha

**β:** beta

**γ:** gamma

**®:** registered trademark

**™:** trademark

**ADCC:** antibody-dependent cellular cytotoxicity

**AFP:** alpha-fetoprotein

**APC:** antigen-presenting cell

**ARG:** arginine

**ATRX:** alpha-thalassemia X-linked intellectual disability

**BBB:** blood brain barrier

**BCL:** B-cell lymphoma

**BCR:** B-cell receptor

**CCL:** CC-chemokine ligand

**CD:** cluster differentiation

**CFU:** colony-forming unit

**CLR:** C-type Lectin Receptor

**CNS:** central nervous system

**$CO_2$:** carbon dioxide

**ConcA:** concanavalin A

**CRP:** C-reactive protein

**CTL:** cytotoxic T lymphocyte

**CTLA-4:** cytotoxic T lymphocyte-associated protein 4

**DAMP:** danger-associated molecular pattern

**DC:** dendritic cell

**DMEM:** Dulbecco's Modified Eagle Medium

**DMSO:** dimethyl sulfoxide

**DNA:** deoxyribonucleic acid

**DR5:** death receptor 5

**EGFR:** proto-oncogene epidermal growth factor receptor

**ELISA:** enzyme-linked immunosorbent assay

**EP:** extracellular products

**FASL:** FAS ligand

**FBS:** fetal bovine serum

**FGL2:** fibrinogen-like protein 2

**FOXP3:** forkhead box protein 3

**GATA3:** GATA-binding protein 3

**GBM:** Glioblastoma multiforme

**GC:** glucocorticoid

**GITR:** glucocorticoid-induced tumour necrosis factor receptor family related gene

**GM-CSF:** granulocyte macrophage colony-stimulating factor

**HCC:** hepatocellular carcinoma

**HD:** healthy donor

**HLA:** human leukocyte antigen

**HMW:** high molecular weight

**IC:** immune complex

**IDH:** isocitrate dehydrogenase

**IDO:** indoleamine 2,3-dioxygenase

**IFN:** interferon

**Ig:** immunoglobulin

**IL:** interleukin

**IL-1ra:** IL-1 receptor antagonist

**IR:** incidence rate

**iTreg:** induced Treg

**kDa:** kilodalton

**KIR:** killer-cell immunoglobulin-like receptor

**LAG-3:** lymphocyte-activation gene 3

**LLT1:** lectin-like transcript 1

**LMW:** low molecular weight

**LPS:** lipopolysaccharides

**MBP:** mannan-binding protein

**M-CSF:** macrophage colony-stimulating factor

**mDC:** myeloid dendritic cell

**MDSC:** myeloid-derived suppressor cell

**MGMT:** Methylation of the $O^6$-methylguanine-DNA-methyltransferase

**MHC:** major histocompatibility complex

**MICA/B:** MHC class I chain–related antigens A and B

**MOI:** multiplicity of infection

**MR:** mannose receptor

**nAFP:** cord blood–derived normal alpha-fetoprotein

**NK:** natural killer cell

**NKG2D:** natural killer group 2D

**NLR:** nucleotide oligomerization domain-Like Receptor

**NO:** nitric oxide

**nTreg:** natural Treg

**OS:** overall survival

**PAMP:** pathogen-associated molecular pattern

**PBMC:** peripheral blood mononuclear cell

**PD-1:** programmed cell death protein 1

**pDC:** plasmacytoid dendritic cell

**PD-L1:** programmed death-ligand 1

**PenStrep:** penicillin-streptomycin

**PGE:** prostaglandin E

**PRR:** pattern recognition receptor

**PTEN:** phosphatase and tensin homolog

**pTreg:** peripherally-derived regulatory T cells

**RAE1:** retinoic acid early transcript 1

**RLR:** retinoic acid-inducible gene I-Like Receptor

**RMPI:** Roswell Park Memorial Institute

**RNA:** ribonucleic acid

**ROR:** transcription factor retinoic acid receptor related orphan receptor

**ROS:** reactive oxygen species

**RT:** radiotherapy

**RTF:** regeneration and tolerance factor

**SMICA/B:** soluble MICA/B

**SNP:** single nucleotide polymorphism

**SR:** scavenger receptor

**STAT3:** signal transducer and activator of transcription 3

**TAA:** tumour-associated antigen

**TA-DC:** Tumour-associated dendritic cell

**tAFP:** tumour-derived alpha-fetoprotein

**Tc:** cytotoxic T cell

**TCR:** T cell receptor

**TERT:** telomerase reverse transcriptase

**Tfh:** T follicular helper cell

**TGF:** transforming growth factor

**Th:** T helper cell

**$T_H3$:** TGF-β producing T helper 3

**TIL:** tumour infiltrating lymphocyte

**TIM:** T cell immunoglobulin mucin

**TLR:** toll-like Receptor

**TME:** tumour microenvironment

**TMZ:** temozolomide

**TNF:** tumour necrosis factor

**TP53:** tumour protein P53

**$T_R1$:** IL-10–producing type 1 regulatory cells

**TRAIL:** TNF-related apoptosis-inducing ligand

**TRAILR:** TNF-related apoptosis-inducing ligand receptors

**Treg:** regulatory T cell

**tTreg:** thymus-derived regulatory T cells

**ULBP:** UL16-binding protein

**v/v %:** volume/volume percent

**VEGF:** vascular endothelial growth factor

**WHO:** World Health Organization

# INTRODUCTION

# I. INTRODUCTION

Cancer is characterized by an abnormal mass of tissue, with survival and growth advantage, resulting in excessive proliferation. Malignant tumours have the capacity to invade and destroy adjacent structures, but can also spread to distant sites (metastasize) and lead to death (Kumar, V. *et al.* 2014).

Carcinogenesis is the process that leads to the transformation of a normal cell into a neoplastic cell and is divided into three stages: initiation, promotion and progression. Tumour initiation involves exposure of normal cells to chemical, physical or biological carcinogens. These carcinogens cause genetic damage to deoxyribonucleic acid (DNA), providing proliferative advantage of initiated cells relative to surrounding normal cells. Tumour promotion is characterized by a proliferation of the initiated cells to a greater extent than normal cells, increasing the probability of additional genetic damage. Progression is the final stage of neoplastic transformation, where genetic and phenotypic changes and cell proliferation occur. This involves a fast increase in the tumor size, where the cells may undergo further mutations with invasive and metastatic potential (Loeb, L.A. and Harris, C.C. 2008).

Genetic and environmental factors contribute to the development of cancer, however environmental factors appear to be dominant for most cancer (Kumar, V. *et al.* 2014). Cancers caused by environmental agents frequently occur in tissues with the greatest surface exposure to the external environment such as lung cancer, gastrointestinal tract and skin (Loeb, L.A. and Harris, C.C. 2008). Age also has an important influence on the probability of developing a tumour. This effect can be explained by the fact that there is an accumulation of somatic mutations or a decline in immune competence, which accompanies aging (Kumar, V. *et al.* 2014).

Cancer is the second most common cause of death following cardiovascular diseases, causing over 8 million deaths worldwide in 2013 (Fitzmaurice, C. *et al.* 2015). Lung, prostate, colorectal, stomach, and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix uterine, and stomach cancer are the most common among women (Ervik, M. *et al.* 2016). The treatment of patients with cancer has been based on a combination of treatment modalities: surgery, radiotherapy and chemotherapy (Balis, F.M. 1998). The main objective of this treatment is a cure and secondly prolong life and symptom relief (palliate) when the cure is not possible due to advanced disease (Khan, F.A. *et al.* 2005).

Tumour initiation and progression is a complex process that depends on several interactions between the tumour and surrounding tissues. *Hanahan and Weinberg* have identified a set of changes in cellular process that constituted essential properties or hallmarks of cancer. These hallmarks include sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, deregulation of cellular energetics, avoidance of immune destruction, tumour-promoting inflammation, genomic instability and mutation, and activation of invasion and metastasis. In 2011, Hanahan and Weinberg recognize that the immune system plays a critical role in the pathogenesis of cancer since tumours can avoid detection by the immune system and limit the extent of immune destruction by disrupting the processes of the cancer immunity cycle throughout the body (Hanahan, D. and Weinberg, R.A. 2011).

## 1. Overview of the immune system

The immune system has a complex architecture comprised of primary lymphoid organs (bone marrow and thymus) and secondary lymphoid organs (lymph nodes, spleen, Peyer's patches, and mucosal tissues) and a variety of cells that interact and communicate via chemical conductors in order to orchestrate an immune response against foreign substances, as external aggressions of antigenic nature (macromolecules or microorganisms) and internal aggressions (transformed or tumour cells), keeping the homeostatic balance (Chaplin, D.D. 2006; Boehm, T. and Bleul, C.C. 2007; Arosa, F.A. *et al.* 2012; Elsabahy, M. and Wooley, K.L. 2013; Abbas, A.K. *et al.* 2014). A fundamental property of the immune system is its ability to distinguish self from non-self, a condition which is referred to as the "process of immunological tolerance" or self-tolerance (Chaplin, D.D. 2010; Schwartz, R.H. 2012).

Vertebrate immune system has two phases of response determined by the speed and specificity of the reaction: the innate and the adaptive immune response (Parkin, J. and Cohen, B. 2001). The innate and adaptive immune systems are often described as contrasting separate arms of the host response; however, they must act together and in a very coordinated fashion in order to ensure host protection from disease. This division helps us to understand the physiology of an immune response (Chaplin, D.D. 2010).

Blood cells, which are categorized in either the myeloid or the lymphoid lineage, are generated from hematopoietic stem cells (Arosa, F.A. *et al.* 2012). Myeloid lineage cells include megakaryocytes and erythrocytes as well as monocytes (can be differentiated into macrophages) and granulocytes (neutrophils, basophils and eosinophils). Lymphoid

lineage cells include T, B and natural killer (NK) cells. Dendritic cells (DCs) are not clearly grouped either in lymphoid or myeloid lineage, because can arise from either common lymphoid progenitors or common myeloid progenitors (Kondo, M. 2010; Arosa, F.A. *et al.* 2012; Elsabahy, M. and Wooley, K.L. 2013).

## 1.1. Innate immunity

Innate immunity (also called natural or native immunity) is the first line of defense against pathogens or other environmental threats, responding in a short period of time within minutes to hours, playing a crucial role in the early recognition and subsequent triggering of a protective inflammatory response to invading pathogens (Alberts, B. *et al.* 2002; Mogensen, T.H. 2009). Moreover, innate immunity plays a central role in activating and modulating the subsequent adaptive immune response (Ezekowitz, R.A.B. and Hoffmann, J.A. 2002; Turvey, S.E. and Broide, D.H. 2010).

The primary function of innate immunity is the recruitment of immune cells to sites of infection and inflammation through the production of cytokines (small proteins involved in cell-cell communication) (Warrington, R. *et al.* 2011).

The main components of innate immunity (figure 1) include (1) physical and chemical barriers, such as epithelia and antimicrobial chemicals produced at epithelial surfaces; (2) effector cells such phagocytic cells (neutrophils and macrophages), DCs, NK cells and other innate lymphoid cells; (3) mechanisms of pattern recognition (Toll-like receptors); and (4) humoral mechanisms, such as members of the complement system and other mediators of inflammation (cytokines that regulate the function of other cells, chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, and bioactive amines and enzymes) (Chaplin, D.D. 2006; Abbas, A.K. *et al.* 2014; Raval, R.R. *et al.* 2014).

Tissue-resident innate immune cells, such as DCs, macrophages, mast cells or even epithelial cells have surface and intracellular receptors that recognize evolutionarily conserved structures on pathogens, known as pathogen-associated molecular patterns (PAMPs) (Arosa, F.A. *et al.* 2012; Madureira, P. *et al.* 2015). This recognition is done through a limited number of germ line-encoded pattern recognition receptors (PRRs). PRRs are found on practically all cells of the body and can be expressed as surface, intracellular or secreted (decoy) receptors. There are several classes of PRRs, including Toll-like Receptors (TLRs), C-type Lectin Receptors (CLRs), Retinoic acid-inducible gene I-Like Receptors (RLRs), Nucleotide oligomerization domain-Like Receptors (NLRs) and cytosolic DNA sensors (Thompson, M.R. *et al.* 2011). Ligand-binding to PRRs results in production

of a variety of pro-inflammatory and antiviral cytokines and chemokines, subsequently inducing adaptive immune responses (Mogensen, T.H. 2009; Murphy, K.M. 2011; Cui, J. *et al.* 2014). The same PRR that engage pathogen-associated molecules are also able to recognize stress-related self-molecules, so-called danger-associated molecular patterns (DAMPs). DAMPs include altered proteins such as β-amyloid fibres, heat-shock proteins, cholesterol and uric acid deposits. Both, PAMPs and DAMPs are recognized by our immune system and induce strong inflammatory responses - danger signals (Madureira, P. *et al.* 2015).

The important ability of the innate immune system to recognize and limit microbes early during infection is based primarily on employment of complement activation, phagocytosis, autophagy, and immune activation by different families of PRRs (Mogensen, T.H. 2009).

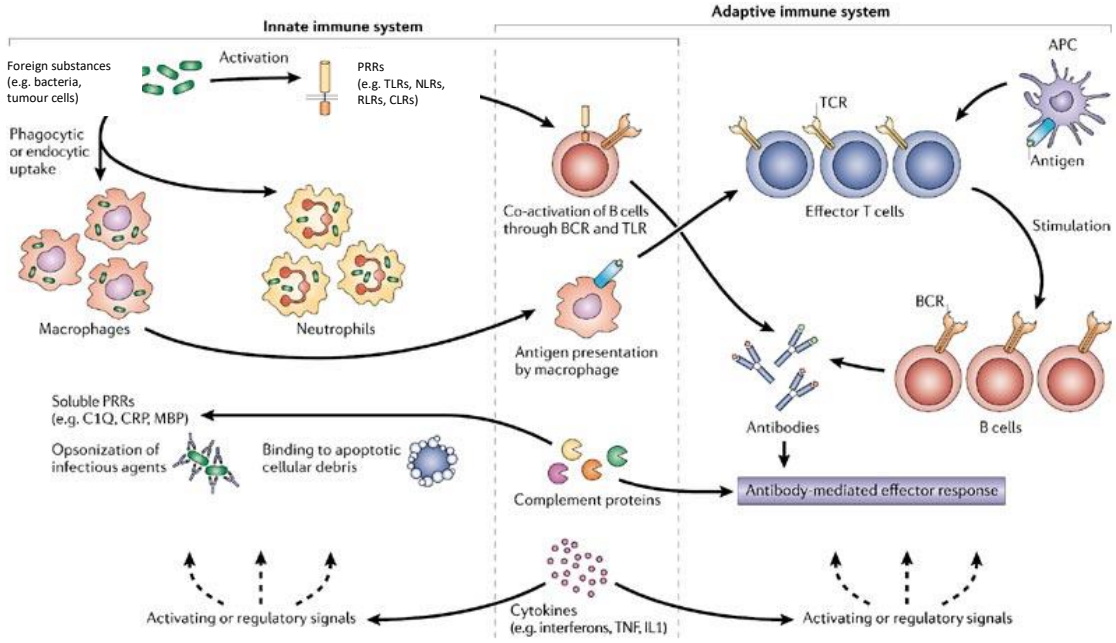


**Figure 1 - The innate and adaptive immune systems and the overlap between them.** Innate immunity is the first line of defence and involve nonspecific responses to foreign infectious agents. These include cellular functions such as phagocytosis and endocytosis by macrophages and neutrophils. Innate immunity plays a central role in activating and modulating the subsequent adaptive immune response through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Adaptive immune mechanisms involve the engagement of receptors that are selected for reactivity with specific antigens (T-cell receptors (TCRs) and immunoglobulin receptors on B cells). The full development of these responses requires the expansion and differentiation of the specific responder cells, which establishes a memory for the specific antigen response. (Adapted from: Gregersen, P.K. and Behrens, T.W. 2006). APC, antigen-presenting cell; BCR, B-cell receptor; CLR, C-type Lectin Receptors; CRP, C-reactive protein; IL, interleukin; MBP, mannan-binding protein; NLR, NOD-like receptors; PPR, pattern recognition receptor; RLR, RIG-I-like receptor; TLR, toll-like receptor; TNF, tumour necrosis factor.

Neutrophils, monocytes, DCs and macrophages are commonly designated as “professional” phagocytic cells and play a crucial role in host defense due to their ability to recognize and eliminate invading pathogens (Steevels, T.A. and Meyaard, L. 2011).

Phagocytic cells produce reactive oxygen species (ROS), inflammatory cytokines, and chemokines, leading to bacterial killing and to recruitment and activation of additional immune cells (Steevels, T.A. and Meyaard, L. 2011).

### 1.1.1. Macrophages

Macrophages are antigen-presenting cells (APC) that play a central role in the clearance of invading pathogens by phagocytosis, production of ROS and nitrogen species and instruction of the other immune cells (Linehan, E. 2015). They are derived from monocytes that differentiate from monoblasts in the bone marrow. Upon tissue damage or infection, monocytes are rapidly recruited to the tissue, where differentiate into mature macrophages (Murray, P.J. and Wynn, T.A. 2011; Yang, J. *et al.* 2014).

Macrophages are highly plastic cells, which allows them to polarize in different phenotypes (figure 2) according to signals produced in the local microenvironment: classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Genin, M. *et al.* 2015; Linehan, E. 2015).

Bacterial cell wall components such as lipopolysaccharides (LPS), interferon (IFN)-γ produced by NK, T helper 1 (Th1) cells, granulocyte macrophage colony-stimulating factor (GM-CSF) and TLR agonists polarize macrophages towards the M1 phenotype (Chanmee, T. *et al.* 2014; Martinez, F.O. and Gordon, S. 2014; Genin, M. *et al.* 2015). Upon activation, M1 macrophages produce proinflammatory cytokines like tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-12, nitric oxide (NO) and reactive oxygen intermediates, pro-inflammatory chemokines and express high levels of the major histocompatibility complex (MHC) class I and class II molecules. M1 phenotype drives pro-inflammatory, cytotoxic and anti-tumour responses (Chanmee, T. *et al.* 2014; Wang, N. *et al.* 2014).

In contrast, M2 macrophage polarization can be induced by IL-4 and IL-13, TLRs and immune complexes, IL-1 receptor ligands, IL-10, macrophage colony-stimulating factor (M-CSF), transforming growth factor (TGF)-*β*, and glucocorticoids (Hao, N.B. *et al.* 2012; Martinez, F.O. and Gordon, S. 2014; Genin, M. *et al.* 2015). M2 macrophages express high levels of scavenger receptor, mannose receptor, IL-1 receptor antagonist, and IL-1 decoy receptor and produce a high level of IL-10, which lead M2 macrophages to mainly promote angiogenesis, immunosuppression, and tumour progression (Hao, N.B. *et al.* 2012).

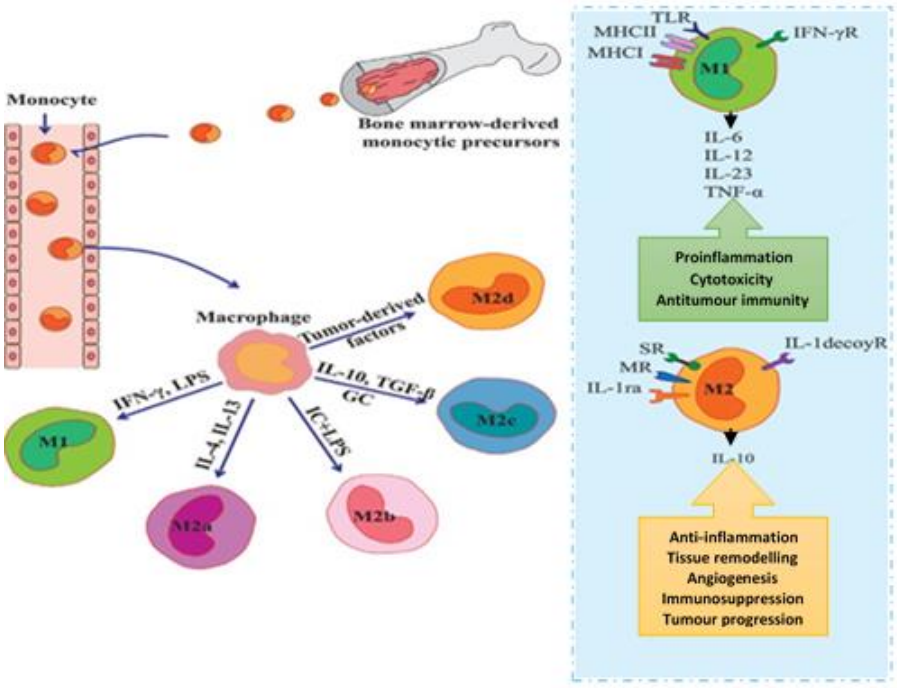


**Figure 2 - Macrophage polarization and its function.** Macrophages can polarize into M1 (also known as classically activated) and M2 (also known as alternatively activated) macrophages that have distinct functional phenotypes. M2 macrophages do not constitute a uniform population and often are further subdivided into M2a, M2b, M2c and M2d categories. The common denominator of all four subpopulations is high IL-10 production characteristic of its immunosuppressive function. (Adapted from: Chanmee, T. et al. 2014). GC, glucocorticoid; IC, immune complex; IFN, interferon; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharides; MHC, major histocompatibility complex; MR, mannose receptor; SR, scavenger receptor; TGF, transforming growth factor; TLR, toll-like Receptor; TNF, tumour necrosis factor.

### 1.1.2. Dendritic cells

Dendritic cells (DCs), a class of bone-marrow-derived cells, are known as APCs "professional" and have the ability of linking the innate and adaptive immune systems. This cells play a key role in inducing and maintaining the anti-tumour immunity, however in the tumour environment their antigen presenting function may be impaired (Ma, Y. et al. 2013). Immature DCs are highly reactive to molecules released by damaged tissues (uric acid), products derived from pathogens (LPS, bacterial DNA and viral RNA) and inflammatory cytokines (TNF-α, IL-1 and IL-2). In the presence of these stimuli, DCs undergo a phenotypic and functional maturation process (Arosa, F.A. *et al.* 2012; Abbas, A.K. *et al.* 2014). Upon phagocytosis and peptide processing, mature DCs display different types of antigens, including tumour antigens, via MHC class I or class II molecules to naïve T cell, for generate robust antigen-specific T cell immune responses. There are two major subsets of DCs: myeloid DCs (mDCs; also known as conventional DCs and classical DCs) and plasmacytoid DCs (pDCs). Tumour-associated DCs (TA-DCs) is another subtype of DCs identified in the tumour microenvironment (TME) with associated immunosuppressive

responses (Palucka, K. and Banchereau, J. 2012; Ma, Y. *et al.* 2013; Mantia-Smaldone, G.M. and Chu, C.S. 2013; Abbas, A.K. *et al.* 2014).

DCs can be utilized as therapeutic cancer vaccines in order to induce tumour-specific effector T cells that can induce tumour regression and/or eradication and can induce immunological memory to control tumour relapse. Typically, DCs are isolated from the patient's blood, and then are stimulated in the lab with cancer antigens and cytokines. After this the cells are injecting back into the patient in order to stimulate the immune response (Palucka, K. and Banchereau, J. 2012; Mantia-Smaldone, G.M. and Chu, C.S. 2013). The potential role of DCs in cancer immunotherapy have expanded remarkably, and there is a new array of therapeutic options (figure 3).

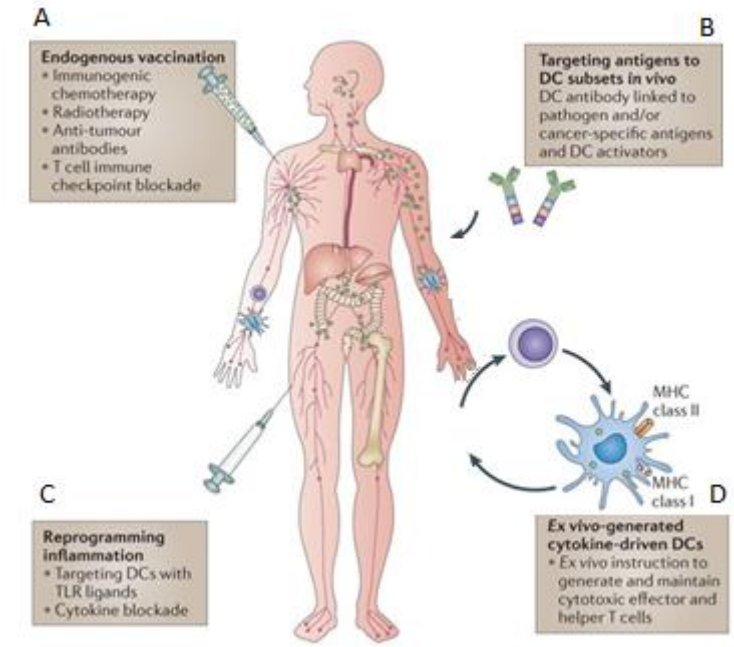


**Figure 3 - Therapeutic options with DCs.** A| The endogenous vaccination with dendritic cells results from in vivo antigen release owing to immunogenic cell death in response to chemotherapy, radiotherapy and immunomodulation approaches that are targeted at T cells. B| Antigens can be directly delivered to DCs in vivo using chimeric proteins that are comprised of an antibody that is specific for a DC receptor fused to a selected antigen. C| Targeting DCs in the tumour microenvironment to reprogramme pro-tumour inflammation leading to tumor rejection D | Vaccines can be based on ex vivo-generated tumor antigen-loaded DCs that are injected back into patients. (Adapted from: Palucka, K. and Banchereau, J. 2012) DC, dendritic cells; MHC, major histocompatibility complex; TLR, toll-like receptor.

### 1.1.3. Natural killer cells

NK cells were first identified in 1975 as large granular lymphocytes with the ability to eliminate certain types of tumour cells without prior activation (Arosa, F.A. *et al.* 2012; Cheng, M. *et al.* 2013). Nowadays, there is a better understanding of the biology and function of NK cells. These cells develop in bone marrow from common lymphoid

progenitor cells, and are defined as $CD3^{-}CD56^{+}$ lymphocytes, which are distinguished as $CD56^{bright}$ and $CD56^{dim}$ subsets (Caligiuri, M.A. 2008; Cheng, M. *et al.* 2013).

NK cells are characterized by the absence of a TCR and its activation dependent on the balance between inhibitory and activating signals from invariant receptors. Activation of NK cells results in the secretion of cytokines that regulate the innate (phagocytosis, antigen presentation) and adaptive (differentiation of T and B cells) immune response, and the elimination of target cells by cytotoxic process, which can involve the production of granules cytotoxic, as well as the aggregation of receptors that induce cell death (Arosa, F.A. *et al.* 2012; Eguizabal, C. *et al.* 2014).

NK cells play critical roles in the early control of viral infection, in hematopoietic stem cell transplantation, in reproduction and in tumour immunosurveillance (Cheng, M. *et al.* 2013).

During tumour progression, tumour cells develop several mechanisms to escape control from NK cells, such as the loss of expression of adhesion molecules, costimulatory ligands or ligands for activating receptors, the upregulation of MHC class I, soluble MIC, FAS ligand (FASL) or NO expression, the secretion of immunoregulatory molecules or immunosuppressive modulators such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E (PGE) 2, and TGF-β . Several abnormalities of NK cells have been observed in cancer patients, including overexpression of inhibitory receptors, decreased cytotoxicity, defective expression of activating receptors or intracellular signalling molecules, defective proliferation, decreased numbers in peripheral blood and in tumour infiltrate, and defective cytokine production, opening new avenues for NK-based immunotherapeutic approaches (Waldhauer, I. and Steinle, A. 2008; Cheng, M. *et al.* 2013; Chretien, A.S. *et al.* 2014; Eguizabal, C. *et al.* 2014).

### 1.1.4. Gamma delta (γδ) T cells

γδ T cells represent a small subset of T cells that express a distinct TCR composed of γδ chains, not requiring the help of conventional MHC class I and class II molecules for recognizing the antigens (Bonilla, F.A. and Oettgen, H.C. 2010). γδ T cells bridge innate and adaptive immunity, through a wide range of defense mechanisms against microorganisms and diseases like cancer (Bonilla, F.A. and Oettgen, H.C. 2010; Gogoi, D. and Chiplunkar, S.V. 2013).

In the tumour environment, there is an accumulation of metabolites of the mevalonate pathway – phosphoantigens - which are a powerful danger signal for the immune system,

leading to γδ T cells activation in a TCR dependent manner (Gogoi, D. and Chiplunkar, S.V. 2013). Human γδ T cells can act as professional APC because they can process and display antigens and can also provide co-stimulatory signals necessary for the induction of proliferation, differentiation and target cell killing. The activation of effector γδ T cells leads to production of the anti-tumour cytokines, such as IFN-γ and TNF-α; induction of apoptosis of transformed cells through engagement of death-inducing receptors as FAS, TNF-related apoptosis-inducing ligand receptors (TRAILR); and release of perforins and granzymes that directly kill tumour cells. In the presence of anti-tumour cell monoclonal antibodies, γδ T cells express CD16 promoting antibody-dependent cellular cytotoxicity (ADCC). γδ T cells can induce a robust NK cell-mediated anti-tumour cytotoxicity through engagement of the CD137 pathway (Bonneville, M. *et al.* 2010; Braza, M.S. and Klein, B. 2013).

Intratumoral γδ T cell infiltration has a variable prognostic value: from positive (melanoma) to neutral (renal cancer) to negative (breast cancer and colorectal cancer) correlations with patient outcomes (Silva-Santos, B. *et al.* 2015).

## 1.2. Adaptive immunity

In contrast to innate immunity, adaptive immunity is a late immune response, but manifests exquisite specificity for its target antigens by virtue of the antigen-specific receptors expressed on the surfaces of T and B lymphocytes (figure 1). Another characteristic of adaptive immunity is the ability to manifest immune memory, ensuring a more vigorous response against specific pathogens or toxins when they are encountered a second time. Immunological memory can persist even decades after the initial sensitizing encounter. The principal cells of the adaptive immune system are lymphocytes and their secreted products (such as antibodies) (Chaplin, D.D. 2006; Chaplin, D.D. 2010;Abbas, A.K. *et al.* 2014).

Adaptive immunity is divided into two types of immune responses: humoral immunity and cellular immunity. Humoral immunity is involved in the eradication of microbes present in the blood or fluid by generating antibodies, which are produced by B cells. On the other hand, cellular immunity is mediated by T cells, and is responsible for the eradication of cancer cells and microbes hidden inside cells (Akira, S. 2011; Abbas, A.K. *et al.* 2014).

### 1.2.1. T cells and cellular immunity

T cells derive from hematopoietic stem cells in bone marrow and, following migration, mature in the thymus. These cells express a unique antigen-binding receptor on their membrane, known as the T-cell receptor (TCR), which requires the action of APCs (usually

dendritic cells, but also macrophages, B cells, fibroblasts and epithelial cells) to recognize a specific antigen (Warrington, R. *et al.* 2011).

T cells recognize peptide fragments of antigens that have been taken up by APCs through the process of phagocytosis or pinocytosis (Chaplin, D.D. 2010). APCs engulf and digest foreign antigens and present fragments of the antigens associated with surface-exposed MHC molecules (also designated human leukocyte antigen or HLA) to T cells. There are two classes of MHC molecules that present fragments of proteins that either have been synthesized within the cell (class I MHC molecules) or that have been ingested by the cell and proteolytically processed (class II MHC molecules). Unlike MHC class I expression, which is constitutive in all nucleated cells, MHC class II molecules are present on APCs where its expression is up-regulated by innate immune stimuli, including ligands for TLRs (Chaplin, D.D. 2006).

Interaction between the TCR and peptide-MHC provides only a partial signal for cell activation. Full activation requires the additional interaction between the costimulatory molecule CD28 on the T cell and CD80 or CD86 (also designated B7.1 and B7.2, respectively) on the APC (Chaplin, D.D. 2006). The MHC-antigen complex activates the TCR and the T cell secretes cytokines which further control the immune response. This antigen presentation process stimulates naïve $CD4^+$ or $CD8^+$ T cells to differentiate into either T helper (Th) cells ($CD4^+$ cells) or cytotoxic T (Tc) cells ($CD8^+$ cells), depending on the cytokines and costimulatory signals they receive during the activation process (Chaplin, D.D. 2006; Warrington, R. *et al.* 2011).

## T-cell effector subsets

**$CD8^+$ T cells** represent a major fraction of circulating T cells and are important in the defence against intracellular pathogens and transformed cells. Once activated, naïve $CD8^+$ T proliferate and can differentiate into effectors (cytotoxic T lymphocytes or CTLs) and memory $CD8^+$ T cells. The different subsets of $CD8^+$ T cells are shown in figure 4 (Bonilla, F.A. and Oettgen, H.C. 2010; Murphy, K.M. 2011). These cells can be identified by differential expression of cell surface markers including CD3 and CD8 and IFN-$\gamma$ production. CTLs are responsible for the direct killing of infected, damaged, and dysfunctional cells, including tumour cells, by secretion of perforin and granzymes, which cause apoptosis of target cells (Dong, C. and Martinez, G.J. 2010).

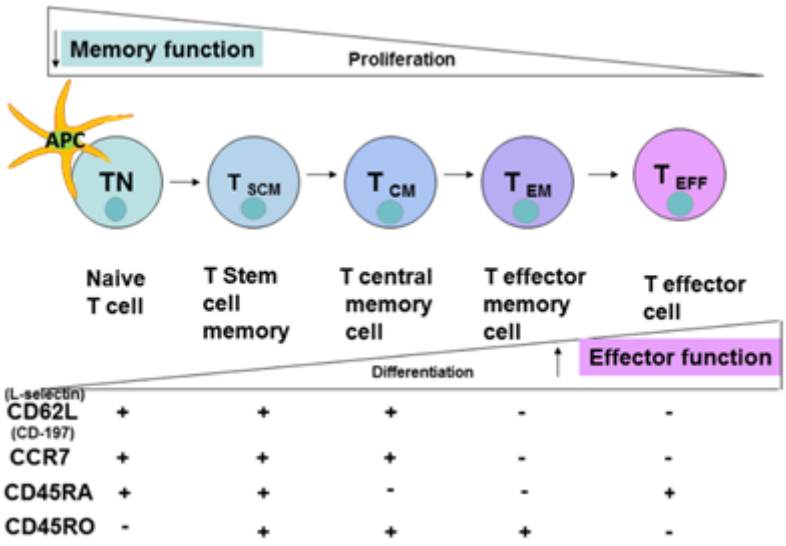


**Figure 4 - The differentiation of CD8⁺ T cells and different CD8⁺ subsets.** Naïve T cells differentiate into stem cell memory cells ($T_{SCM}$), T Central Memory cells, ($T_{CM}$), T effector memory cells ($T_{EM}$), and T effector cells ($T_{EFF}$). L-Selectin, CD45RO, CD45RA and CCR-7 are the different CD8⁺ markers upon cell differentiation. The memory function and proliferation are decreased, while the effector function is increased upon CD8+ T cell differentiation (Adapted from: Golubovskaya, V. and Wu, L. 2016).

**CD4⁺ T cells or T helper (Th) cells** differentiate into several subsets of effector T cells with a variety of different functions. The main functional classes are Th1, Th2, Th17, T follicular helper (Tfh) and regulatory T (Treg) cells, which are characterized by different cytokine profiles and have different functions (figure 5) (Murphy, K.M. 2011). CD4⁺ αβ TCR population have little cytotoxic activity and secrete cytokines that act on other leucocytes such as B cells, macrophages, eosinophils, or neutrophils to clear pathogens (Bonilla, F.A. and Oettgen, H.C. 2010). Th can be identified by differential expression of cell surface markers including CD3 and CD4 (Elsabahy, M. and Wooley, K.L. 2013).

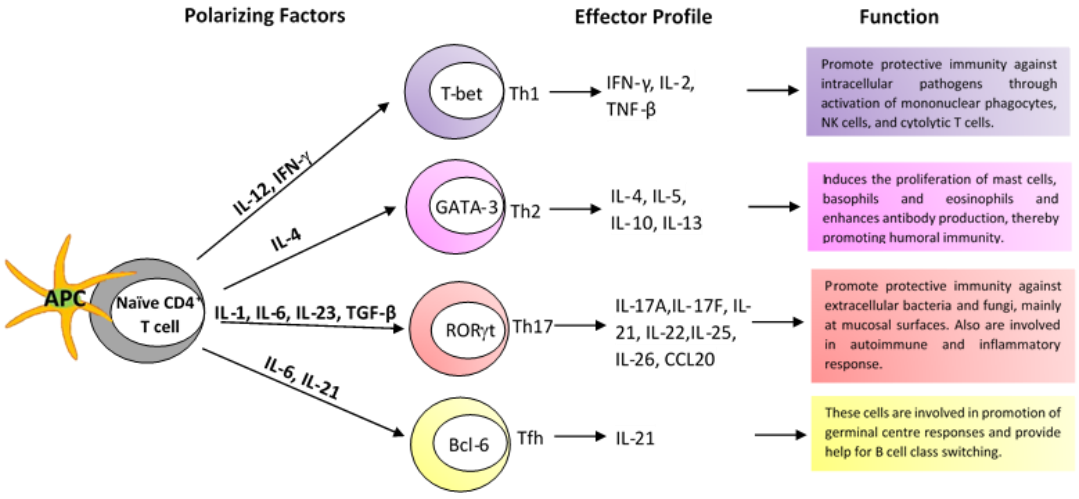


**Figure 5 - Different subsets of CD4⁺ T cells.** Naïve CD4⁺ T cells can differentiate into distinct effector T-helper and regulatory T-cell subsets, depending on the nature of antigen stimulation signal received from APCs. The different CD4⁺ subsets are characterized by expression of lineage-specific transcriptional regulators, cell surface

markers, and secretion of key cytokines. (Reprinted from: Bonilla, F.A. and Oettgen, H.C. 2010; Dong, C. and Martinez, G.J. 2010; Murray, P.J. and Wynn, T.A. 2011; Elsabahy, M. and Wooley, K.L. 2013; Ma, C.S. and Deenick, E.K. 2014; Tripathi, S.K. and Lahesmaa, R. 2014). BCL-6, B cell lymphoma 6;CCL, CC-chemokine ligand; FOX, forkhead box; GATA3, GATA-binding protein 3; IFN, interferon; IL, interleukin; ROR, retinoic acid receptor-related orphan receptor; T-bet, T-box expressed in T cells; Tfh, T follicular helper; TGF, transforming growth factor; Th, T helper; TNF, Tumour necrosis factor.

**Regulatory T (Treg) cells**, a small subpopulation of $CD4^+$ T cells, play a crucial role in the maintenance of peripheral immunological tolerance and control of immune responses toward pathogens and tumours. These cells are a heterogeneous population with respect to their origin of development, functional activity and activation status. They use diverse suppressive mechanisms depending on microenvironments and target cells, including inhibitory cytokines (e.g., IL-10, IL-35 and TGF-β), cytolysis, metabolic disruption or the modulation of dendritic cell function (Guo, J. and Zhou, X. 2015). Treg cells are generally categorized into two groups: thymus-derived Treg (tTreg) cells and peripherally-derived Treg (pTreg) cells, also known as natural Treg (nTreg) and induced Treg (iTreg), respectively (figure 6) (Ohkura, N. *et al.* 2013; Li, Z. *et al.* 2015).

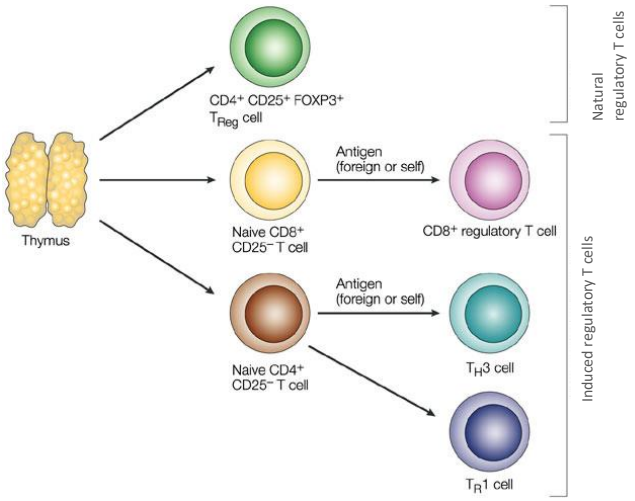


**Figure 6 - Natural and induced regulatory T cells.** Regulatory T cells are broadly classified into natural or induced. Natural Tregs are $CD4^+CD25^+$ T-cells which develop, and emigrate from the thymus to perform their key role in immune homeostasis. Induced Tregs are non-regulatory $CD4^+$ or $CD8^+$ T-cells which acquire CD25 (IL-2R alpha) expression outside of the thymus, and are typically induced by inflammation and disease processes, such as autoimmunity and cancer. (Adapted from: Mills, K.H.G. 2004). CD, cluster differentiation; FOXP3, forkhead box protein 3; $T_H3$, TGF-β producing T helper 3; $T_R1$, IL-10–producing type 1 regulatory cells.

The natural self-antigen-reactive $CD4^+CD25^+$ Treg are generated in the thymus and then enter peripheral tissues, where they suppress the activation of other self-reactive T cells. These cells express the transcription factor forkhead box protein 3 (FOXP3) and highly express surface and intracellular markers, including CD25, glucocorticoid-induced tumour necrosis factor receptor family related gene (GITR) and cytotoxic T lymphocyte-

associated protein 4 (CTLA-4) and mediate immune suppression through a cell–cell contact mechanism (Dasgupta, A. and Saxena, R. 2012; Li, B. and Wang, R.-F. 2012; Okada, M. *et al.* 2014; Guo, J. and Zhou, X. 2015).

Other populations of antigen-specific regulatory T cells can be induced from naïve $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery under the influence of semi-mature dendritic cells, IL-10, TGF-β and possibly IFN-α (figure 7). The inducible populations of regulatory T cells include IL-10–producing type 1 regulatory ($T_R1$) cells and TGF-β producing T helper 3 ($T_H3$) cells (Wan, Y.Y. and Flavell, R.A. 2007; Murphy, K.M. 2011; Li, B. and Wang, R.-F. 2012; Yao, Y. *et al.* 2015). $CD8^+$ regulatory T cells are a subtype $CD8^+$ T cells that secrete either IL-10 or TGF-β, leading to the suppression of the anti-tumour activity of CTLs and NK cells (Mills, K.H.G. 2004).

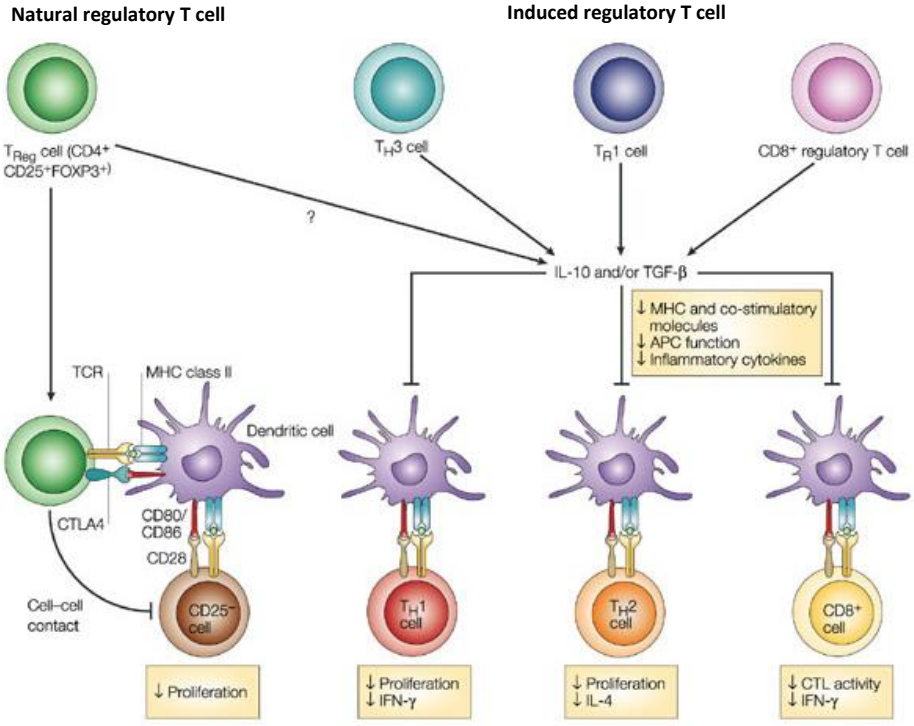


**Figure 7 - Targets of regulatory T cells and mechanisms of suppression**. $CD4^+CD25^+$ Treg cells express: CD25, which required for Treg cell survival (and presumably for IL-2 absorption as part of Treg cell-mediated suppression); CTLA-4, which is involved in the suppressive function of Treg cells by downregulating CD80 and CD86 expression on antigen-presenting cells; and FOXP3, which is essential for Treg cell development and suppressive activity, partly through the maintenance of high levels of CD25 and CTLA-4 expression. These cells also secrete TGF-β or IL-10, which have a role in suppression. $T_R1$ cells, $T_H3$ cells and $CD8^+$ regulatory T cells, secrete IL-10 and/or TGF-β. These immunosuppressive cytokines inhibits antigen-presenting cells by reducing the expression of MHC molecules and costimulatory molecules, and blocks T -cell cytokine production. (Adapted from: Mills, K.H.G. 2004). APC, antigen-presenting cell; CD, cluster differentiation; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte-associated protein 4; FOXP3, forkhead box protein 3; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; TCR,T cell receptor; TGF, transforming growth factor; Th, T helper cell; $T_H3$, TGF-β producing T helper 3; $T_R1$, IL-10–producing type 1 regulatory cells; Treg, regulatory T cell.

Treg cells are found at high frequencies in tumour tissues of various types of cancers. In TME, Treg cells play a significant role in the suppression of anti-tumour immune responses against cancer cells. There is a heterogeneity in tumour-associated Treg cells, suggesting that they are accumulated by different mechanisms such as peripheral recruitment, proliferation of cells in the TME, or differentiation of progenitors in response to tumour-secreted factors (Quail, D. and Joyce, J. 2013). High $FOXP3^+$ Tregs infiltration was significantly correlate with reduced overall survival in some tumour types, including breast cancer, melanoma and hepatocellular carcinoma; whereas, $FOXP3^+$ Tregs were associated with improved survival in colorectal, head and neck, and oesophageal cancers (Quail, D. and Joyce, J. 2013; Shang, B. *et al.* 2015) .

**Th17 cells** are generated in the presence of IL-6 and TGF-β, under the control of the transcription factor retinoic acid receptor related orphan receptor (ROR)γt and signal transducer and activator of transcription 3 (STAT3). These cells secrete IL-17A, IL-17F, IL-21, IL-22 and CC-chemokine ligand (CCL)-20 (Gnerlich, J.L. *et al.* 2010; Ye, J. *et al.* 2013).

Th17 cells are often associated with tumours. Increased levels of tumour infiltrating Th17 cells have been detected in many human cancers, including ovarian, melanoma, breast, pancreatic, renal cell, and gastric cancer. Intratumoral Th17 cell infiltration has a variable prognostic value. In ovarian cancer, prostate cancer, lung carcinoma, and squamous cell carcinoma, Th17 cell infiltration was correlated with better survival, while in hepatocellular, colorectal, pancreatic, and hormone resistant prostate carcinoma patients was correlated with bad prognosis (Gnerlich, J.L. *et al.* 2010; Guery, L. and Hugues, S. 2015).

### 1.2.2. B cells and humoral immunity

The humoral immune response is mediated by antibodies that are secreted by plasma cells that develop from B cells (Bonilla, F.A. and Oettgen, H.C. 2010). B cell development occurs in the bone marrow with gene recombination in the immunoglobulin locus, which results in the surface expression of a unique B cell receptor (BCR) (Batista, F.D. and Harwood, N.E. 2009).

After the recognition of antigens in association with MHC class II molecules by BCR and PRR ligands, $CD4^+$ T-cell help are recruited and naïve mature B cells proliferate and differentiate into effector B cells (plasma cells) or memory B cells (figure 8) (Nothelfer, K. *et al.* 2015). Plasma cells are generated during the primary response and are characterized by the production of antigen-specific immunoglobulins (antibodies), mostly immunoglobulin

M (IgM) (but some IgG) (Gray, D. 2002; Nothelfer, K. *et al.* 2015). Memory B cells are long lived and can quickly be reactivated to differentiate into plasma cells following secondary infection. Together, memory B cells and long-lived plasma cells form the basis for lifelong B cell-mediated protection against infections (Nothelfer, K. *et al.* 2015).

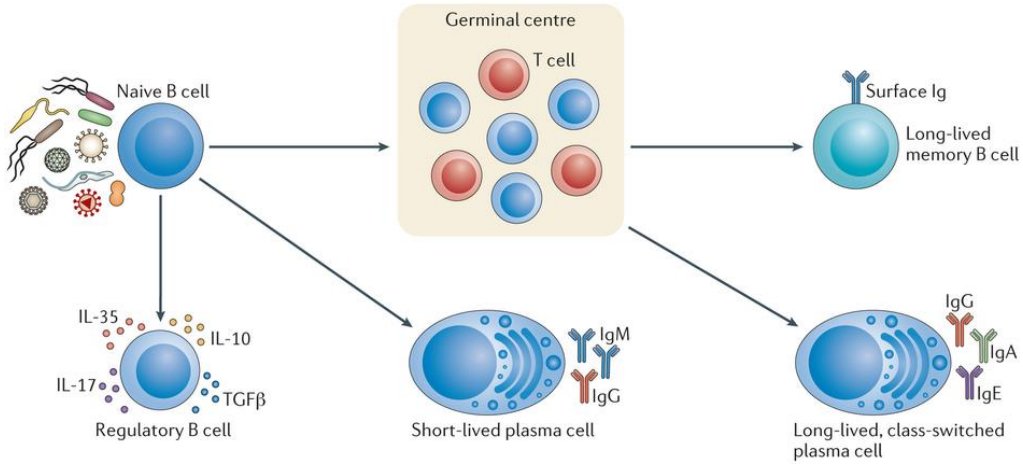


**Figure 8 - B cell responses to infection.** In response to activation signals, naive mature B cells proliferate and differentiate into effector cells. The integration of several infection-related signals, including binding of specific antigens to the B cell receptor (BCR) and pattern recognition receptor (PRR) ligands results on B cell activation. At an early stage, short-lived plasma cells that secrete polyreactive antibodies can be induced. Regulatory B cells can also be generated leading to the production of immunosuppressive cytokines such as IL-10, IL-17, IL-35 and TGF-β, which modulate T cell responses. Sustained B cell activation leads to further differentiation and selection in organized lymphoid structures, called germinal centres. The activation of nuclear factor-κB (NF-κB) and upregulation of activation-induced cytidine deaminase (AID) induce affinity maturation of antibodies through somatic hypermutation and class-switch recombination of the antibody heavy chain. This ultimately results in the differentiation of specific, long-lived plasma cells and memory B cells, which confer protective immunity. (Adapted from: Nothelfer, K. et al. 2015). Ig, immunoglobulin; IL, interleukin; TGF, transforming growth factor.

# 2. Cancer and the immune system

After years of controversy, it is now accepted the hypothesis that the immune system influence the development and biology of cancer. Nowadays, there is a clearer understanding of the ways by which tumour cells escape the host response, leading Hanahan and Weinberg (2011) to recognize that the ability to evade immune destruction is an emerging hallmark of cancer. With this, there is the emergence of new therapies that enable the host immune system to recognize and eliminate cancer cells (Kumar, V. *et al.* 2014; Lam, S.S. *et al.* 2015).

## 2.1. Immunosuppressive microenvironment of the tumour

In the 1860s, Rudolf Virchow observed a tumour-promoting function for leukocytes leading him to create the hypothesis that cancer arises from inflammatory sites. The notion

that the immune system could repress tumour development was initially proposed in 1909 by Paul Ehrlich, and almost 50 years later Lewis Thomas and Macfarlane Burnet proposed the so-called *immunosurveillance hypothesis* for cancer that ascribes to the immune system the ability to recognize and eliminate newly emerging cancer cells (Dunn, G.P. *et al.* 2002; Dunn, G.P. *et al.* 2004; Dadi, S. *et al.* 2016). The action of NK cells and $CD8^+$ T cells is chief in this immunosurveillance theory, because they are able to recognize and rapidly kill recently formed cancer cells (Madureira, P. *et al.* 2015).

More recently, the term *cancer immunosurveillance* was refined and extended giving rise to the *immunoediting hypothesis*, which comprises three sequential phases that are termed the "*three Es of cancer immunoediting*:" elimination, equilibrium, and escape (figure 9) (Dunn, G.P. *et al.* 2004). In the elimination phase, nascent transformed cells can be detected and eliminated by innate and adaptive immune system, through activation of immune effector cells, such as NK and T cells and by the release of effector molecules, such as IFN-γ, perforin, FAS/FASL, and TNF-related apoptosis-inducing ligand (TRAIL) (Kim, R. *et al.* 2007; Mittal, D. *et al.* 2014). The equilibrium phase is the next step in cancer immunoediting in which a continuous sculpting of tumour cells produces cells resistant to immune effector cells, leading to the immune selection of tumour cells with reduced immunogenicity (Kim, R. *et al.* 2007). If tumours circumvent immune recognition and/or destruction, they progress from the equilibrium to the escape phase, becoming clinically apparent (Teng, M.W. *et al.* 2015). The escape phase is characterized by the inability of the immune system in eliminating and controlling the proliferation of tumour cells. The cancer may occur as a result of exhaustion of the immune system or when cancer cells acquire phenotypic changes, allowing them to evade or avoid the immune system (Dunn, G.P. *et al.* 2006).

Tumours can evade the immune system by mechanisms of immune tolerance, resulting in inactivation or tolerization of anti-tumour T cells through several mechanisms induced by tumour or accessory cells. Tumour escape of adaptive immunity may result specifically by elimination of T lymphocytes, the functional inactivation by tolerization/anergy mechanisms or by immunosuppressive cytokines or cells, or it may result from the fact that the lymphocytes are not exposed to tumour antigens - "immunological ignorance" (Abbas, A.K. *et al.* 2014).

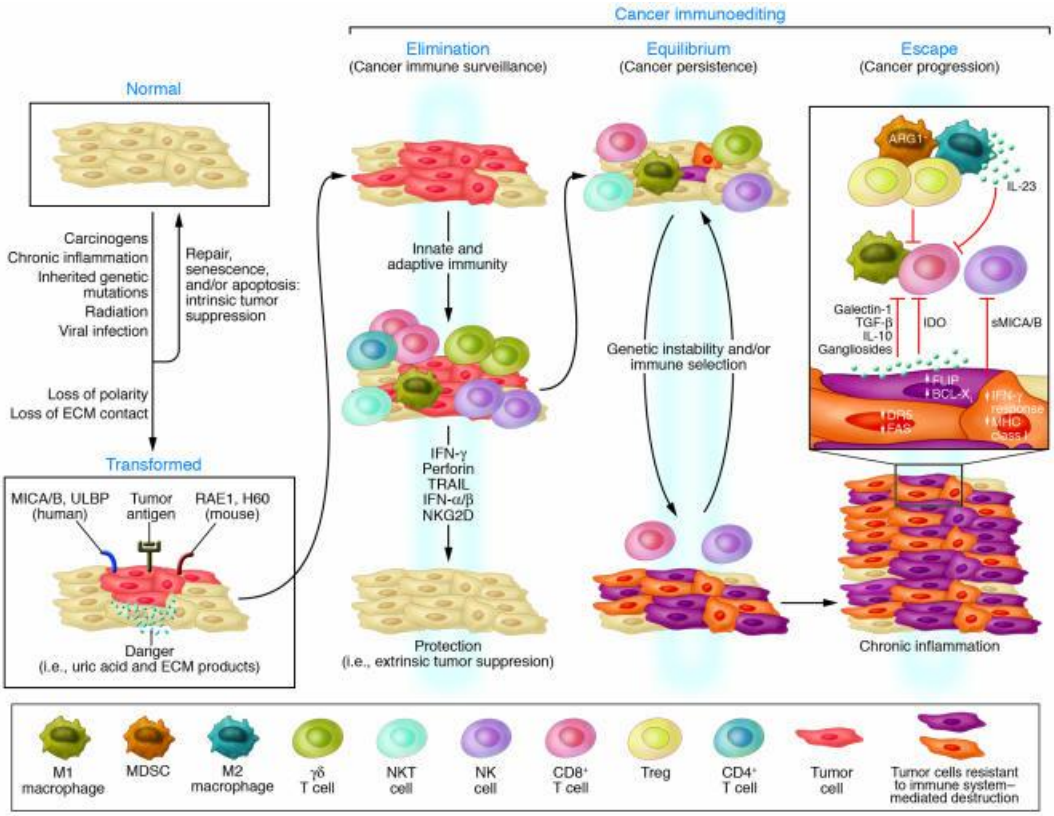


**Figure 9 - The three phases (3 Es) of cancer immunoediting: Elimination, Equilibrium, and Escape.** Tumours may be eliminated in either the elimination or equilibrium phase, leading to protection from tumour growth. However, tumours may ultimately escape immune protection and become clinically manifest in the escape phase. (Adapted from: Swann, J.B. and Smyth, M.J. 2007). ARG, arginine; DR5, death receptor 5; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; MICA/B, MHC class I chain–related antigens A and B; NKG2D, natural Killer group 2D; RAE1, retinoic acid early transcript 1; sMICA/B, soluble MICA/B; ULBP, UL16-binding protein.

However, none of these hypotheses explains why our immune system is not able to recognize and eliminate tumours. The most probable explanation is lacking danger signal. These danger signals are delivered by PAMPs or DAMPs that are recognized by specific receptors at the surface of innate immune cells, triggering an inflammatory response. The tumour rejection occurs when a tumour-associated antigen (TAA) is presented under a danger environment. When a TAA is presented in a context of non-danger, occurs tolerance induction and tumour development (Madureira, P. *et al.* 2015).

There are a large number of mechanisms, which contribute for the high tolerogenic and immunosuppressive microenvironment found in tumours, including: development of an immunosuppressive tumour environment due to the release of cytokines (VEGF, IL-6, IL-10, M-CSF and TGF-β) and immunoregulatory molecules (IDO, CTLA-4, PD-1/PD-L1, Tim-3/ galectin-9, CD39, CD73, LAG-3, arginase, adenosine receptors); loss of tumour antigens, MHC class I or co-stimulatory molecules, which leads to reduced immune

recognition; and increased resistance or survival by increased expression of STAT3 and anti-apoptotic molecule Bcl2 (Mittal, D. *et al.* 2014).

### 2.2. Alpha-Fetoprotein: an example of immunomodulation in cancer

Alpha-fetoprotein (AFP) is an oncofetal protein synthesized in the yolk sac and fetal liver, which is transcriptionally repressed shortly after birth. High levels of AFP are expressed in abnormalities of prenatal development and several epithelial cancers, including hepatocellular carcinoma (HCC). AFP is a TAA which is expressed in about 60% of HCC. It plays a role in the transport of serum components, interfere with intracellular signalling and has an immunoregulatory role (Gonzalez-Carmona, M.A. *et al.* 2006; Pardee, A.D. *et al.* 2014).

Pardee *et al.* reported that AFP exerts immunosuppressive activity inducing dysfunction of DCs. They examined the effects of cord blood–derived normal AFP (nAFP) or HCC tumour-derived AFP (tAFP) on DC differentiation and function. In this study, they showed that tAFP-conditioned DCs expressing reduced levels of DC maturation markers, maintain a monocyte-like morphology and exhibit limited production of inflammatory mediators, limiting the induction of robust T cell proliferative responses. They also observed that full-length AFP protein, along with the presence of low molecular mass copurifying species, is absolutely essential for tAFP to exert its immunosuppressive activity (Pardee, A.D. *et al.* 2014).

Thus, the tAFP can be used as a therapeutic target, through approaches that antagonize its regulatory properties in order to enhance immunity and improve the clinical outcomes (Pardee, A.D. *et al.* 2014).

## 3. Glioblastoma

Gliomas are a common type of primary brain tumour and include astrocytomas, oligodendrogliomas, and ependymomas (Ostrom, Q.T. *et al.* 2015). Gliomas are graded based on histological criteria, nuclear atypia, mitotic activity, endothelial proliferation and the presence of necrosis on a scale of I to IV, defined by the World Health Organization (WHO) (Alifieris, C. and Trafalis, D.T. 2015; Bush, N.A. *et al.* 2016).

Glioblastoma multiforme (GBM), WHO grade IV astrocytoma, is the most common and most aggressive malignant primary brain tumour that develops from the lineage of star-

shaped glial cells, so-called astrocytes (Llaguno-Munive, M. *et al.* 2013; Thakkar, J.P. *et al.* 2014). It constitutes 45.2% of all malignant central nervous system (CNS) tumours and 80% of all primary malignant CNS tumours and develops primarily in the cerebral hemispheres but can develop in other parts of the brain (Bush, N.A. *et al.* 2016). The average annual age-adjusted incidence rate (IR) of GBM is 3.19/100,000 population (CBTRUS, 2013), and a mean age at diagnosis is 64 (Thakkar, J.P. *et al.* 2014). The IR of GBM is 1.5 times higher in males as compared to females, and two times higher in whites than blacks. GBM has a worse prognosis with a median overall survival (OS) about 12–18 months after diagnosis (Bush, N.A. *et al.* 2016). Current standard treatment includes maximal-safe surgical resection followed by radiotherapy (RT) with concurrent temozolomide (TMZ) chemotherapy and adjuvant TMZ for six cycles, depending on the tumour location, the cancer cell type and the grade of malignancy (Thakkar, J.P. *et al.* 2014; Alifieris, C. and Trafalis, D.T. 2015).

The high mitotic activity, microvascular proliferation, and necrosis are characteristic of glioblastoma (Hou, L.C. *et al.* 2006). GBMs are commonly described in two different clinical forms: primary (*de novo*), which correspond to 95% of GBMs, manifest in older patients (mean age 62 years) and are typically characterized by amplification of the proto-oncogene epidermal growth factor receptor (EGFR) gene, mutations in the telomerase reverse transcriptase (TERT) promoter and phosphatase and tensin homolog (PTEN) tumour suppressor gene; and secondary, which develop from prior low-grade astrocytomas or oligodendrogliomas in younger patients (mean age 45 years) and are characterized by mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2), tumour protein P53 (TP53), and alpha-thalassemia X-linked intellectual disability (ATRX). Mutation in IDH1/2 is one of the most important differentiators between primary and secondary GBM (Thakkar, J.P. *et al.* 2014; Alifieris, C. and Trafalis, D.T. 2015).

Methylation of the $O^6$-methylguanine-DNA-methyltransferase (MGMT) promoter has been shown to predict response to alkylating agents such as TMZ, carmustine, and cyclophosphamide (Esteller, M. *et al.* 2002).

GBM is an aggressive disease that progresses rapidly leading to the appearing of various symptoms depending on the size and location of the tumour in the brain. The most common symptoms in patients with GBM are headaches, focal neurologic deficits, confusion, memory loss and personality changes (Alifieris, C. and Trafalis, D.T. 2015).

Etiologically, there are known linked risk factors that lead to development of GBM: genetic factors (neurofibromatosis, Li-fraumeni syndrome, Von hippel-lindau syndrome and Turcot syndrome), environmental factors (prior cranial irradiation and electromagnetic

fields), infections (tuberculosis, varicella zoster and simian virus 40) and occupation exposures (petroleum industry, exposure to pesticides and chemicals, synthetic rubber industry and head trauma) (Schwartzbaum, J.A. *et al.* 2006; Ostrom, Q.T. *et al.* 2014; Alifieris, C. and Trafalis, D.T. 2015). Other factors associated with GBM risk are high socio-economic status, taller height, immune factors and immune genes, as well as some single nucleotide polymorphisms (SNPs) detected by genome wide association studies. A lower risk of gliomas has been associated with allergy or atopic diseases (asthma, eczema, psoriasis) (Thakkar, J.P. *et al.* 2014).

### 3.1. Immunomodulation in Glioblastoma

The CNS was once thought to be an immune-privileged site, devoid of normal immunologic function. The existence of the blood brain barrier (BBB) that allows for selective entry of cells and soluble molecules is one of the main features that support this theory. However, contrary to what was thought, the BBB is not limited to CNS immune cell entry into cancer and other disease. Nowadays, it is know that the CNS has an active and tightly regulated immune system, can elicit vigorous immune-stimulatory as well as immunosuppressive responses. Microglia and monocyte-derived macrophages constitute the largest fraction of glioma-associated myeloid cells. Microglia, the resident macrophages of the CNS, compose 5–20% of the total glial cell population and play an essential role in the innate defense system of the brain. Monocyte-derived macrophages are restricted to perivascular sites choroid and meningeal CNS, and penetrate the parenchyma after disrupted the integrity of BBB due to disease and / or inflammation (Thomas, A.A. *et al.* 2012; Reardon, D.A. *et al.* 2014; Ampie, L. *et al.* 2015; Perng, P. and Lim, M. 2015).

Patients with glioblastoma have a relative systemic immune suppression compared to the general population (Agrawal, N.S. *et al.* 2014). The TME is rich with immunosuppressive factors secreted by the tumour (figure 10), which induce MHC molecule down regulation, production of immunoinhibitory cytokines, polarization of macrophages to the immunosuppressive M2 phenotype, infiltration of immunosuppressive Tregs and impairment of T cell function due to hostile physical factors such as hypoxia (Reardon, D.A. *et al.* 2014; Nduom, E.K. *et al.* 2015).

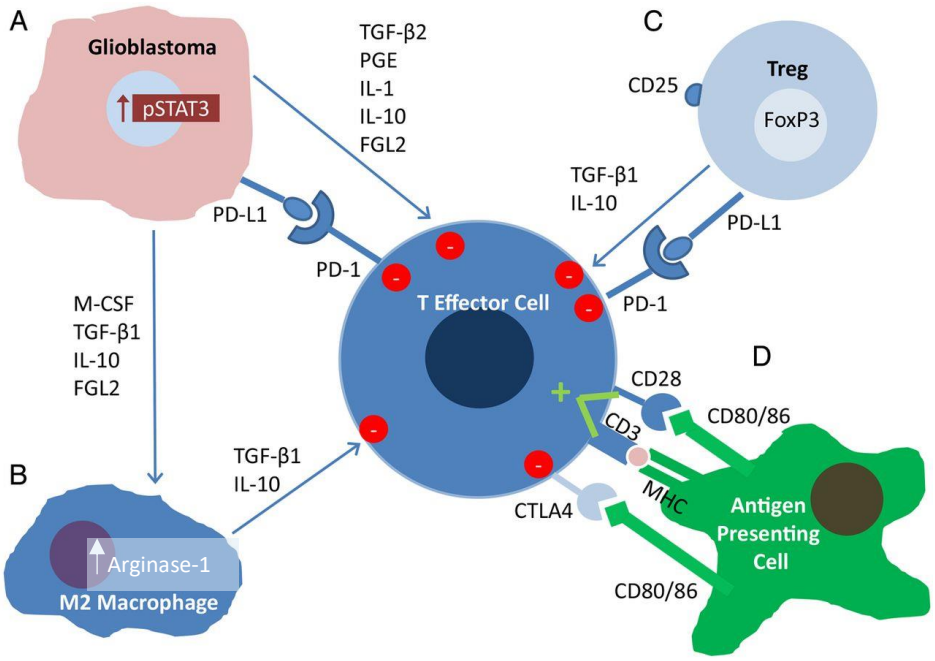


**Figure 10 - Factors contributing to immunosuppression in glioblastoma.** A| Increased expression of STAT3 leads to the production of various immunosuppressive factors such as TGFβ-2, PGE, IL-1, IL-10 and FGL2 by glioblastoma cells, which suppress the activity of effector cells. M-CSF, TGFβ-1 and IL-10 lead to differentiation of macrophages into an M2 phenotype (immunosuppressive). PD-L1 expressed on the surface of glioblastoma cells also engages PD-1 to suppress effector activity. B| M2 Macrophages secrete TGFβ-1 and IL-10, suppressing effector cells further. One of their signatures is production of enzyme Arginase-1 that depletes L-arginine thereby suppressing T cell responses and depriving iNOS of its substrate. C| Regulatory T cells secrete TGFβ-1 and IL-10 as well, further suppressing immune reactivity, while also expressing PD-L1. D| Interaction between the TCR and peptide-MHC provides only a partial signal for cell activation. Full activation requires the additional interaction between the costimulatory molecule CD28 on the T cell and CD80/86. CD80/86 can also suppress activity by engaging the CTLA4 receptor on the activated T cell. (Adapted from: Nduom, E.K. et al. 2015). CD, cluster differentiation; FGL, fibrinogen-like protein; IL, interleukin; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PGE, prostaglandin E; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor; Treg, regulatory T cell.

Glioblastoma cells have an increased expression of STAT3, leading to the release immunosuppressive factors such as TGF-β2, PGE, IL-1, IL-10 and fibrinogen-like protein 2 (FGL2) that suppress the activity of effector cells. FGL2 is a secreted factor that causes immunosuppression by increasing the levels of programmed cell death protein 1 (PD-1) expression, increased number of M2 macrophages, and enhancing the number of myeloid-derived suppressor Cells (MDSCs) and Treg. Tumour cells also express on their surface programmed death-ligand 1 (PD-L1) which binds to PD-1 to suppress effector activity of T cells. M-CSF, TGF-β1 and IL-10 lead to polarization of glioma-infiltrating microglia/tumour associated macrophages toward the immunosuppressive M2 phenotype. M2 macrophages secrete TGFβ-1 and IL-10, suppressing effector cells further. Treg cells suppress immune responses by secreting cytokines, such as TGF-β and IL-10, and by increased expression of PD-L1. APCs presented an antigen in context of MHC molecules, requiring a costimulatory signal from CD80/86 to CD28 for activation. The CTLA-4, which modulates

early stages of T-lymphocyte activation, is constitutively expressed on surface of the activated T cell and acts as a negative regulator on activation of T cells when bound to CD80/86 (Nduom, E.K. *et al.* 2015; Perng, P. and Lim, M. 2015).

Locally at the tumour site, GBM cells can inhibit the anti-tumour effector cell responses by the expression of soluble and membrane proteins described as immunosuppressive molecules (figure 11). Human malignant GBM cells expressing FASL, which binds to FAS expressed on immune effector cells triggering apoptosis of these cells. The CD70 is also expressed on GBM cells, producing an apoptotic effect on CD27-expressing T and B cells. Direct or indirect (via the HLA-E molecule) interaction of HLA-G with inhibitory receptors expressed on cytotoxic lymphocytes results in inhibition of cytotoxicity of NK and T cells, in decreased T cell proliferation and T-cell priming. IDO is a cytosolic enzyme found in gliomas, including GBM, which degrades tryptophan along the kynurenine pathway. Kynurenine facilitates expansion of Tregs populations and inhibition of T cell effector functions. Other receptors expressed on GBM cells are lectin-like transcript 1 (LLT1) and regeneration and tolerance factor (RTF), which inhibit CD161-expressing NK cell cytotoxicity and NK and T-cell-mediated cytotoxicity, respectively (Avril, T. *et al.* 2011; Perng, P. and Lim, M. 2015).

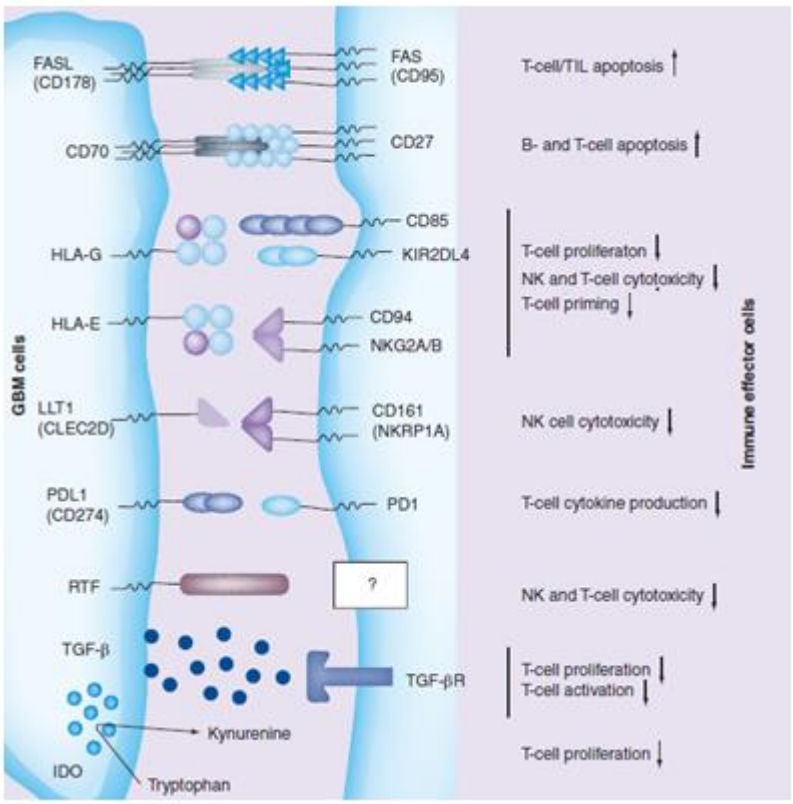


**Figure 11 - Immunosuppressive molecules expressed on glioblastoma multiforme cells, their molecular targets and actions on immune effector cells.** Glioblastoma cells also express soluble proteins (IDO and TGF-β) and membrane proteins (FASL, CD70, HLA-G, HLA-E, LLT1 and PD-L1) described as

immunosuppressive molecules that inhibit the antitumor effector cell responses**.** (Adapted from: Avril, T. et al. 2011). CD, cluster differentiation; FASL ,FAS ligand; GBM, glioblastoma multiforme; HLA, human leukocyte antigen; IDO, indoleamine 2,3-dioxygenase; KIR, killer-cell immunoglobulin-like receptor; LLT1, lectin-like transcript 1; NK, natural killer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; RTF, regeneration and tolerance factor; TGF, transforming growth factor; TIL, tumour infiltrating lymphocyte.

## 3.2. Immunotherapy for the treatment of Glioblastoma

The immune system can be activated by external stimuli to destroy tumour cells effectively, and even to eradicate tumours. In 1891, William Coley observed that some patients with sarcoma undergo spontaneous regression of the tumour when they were injected with heat kills *Streptococcus pyogenes* and *Serratia Marcescen*. This caused an increased activity of the immune system against the tumour by systemic immune activation (Ampie, L. *et al.* 2015; Lam, S.S. *et al.* 2015). This idea boosted for new paths in the treatment of cancer with anti-tumour immunotherapy.

Immunotherapy against cancer is essentially based on the stimulation of the patient's immune system in order to eliminate or neutralize tumour cells and factors that allow tumour survival and proliferation. Compared to conventional therapy, immunotherapy using the components and the mechanisms of the immune system, inducing no damage to normal cells which minimizes side-effects. This therapy has a high therapeutic potential due to various immunological characteristics such as, the cognitive character of acquired immunity (allowing specificity in anti-tumour responses), the establishment of anti-tumour immunologic memory (enabling the prevention of tumour recurrence or keeping it in a state of latency) and the effectiveness of the immune response (Arosa, F.A. *et al.* 2012; Abbas, A.K. *et al.* 2014).

Applying strategies based on innate and adaptive immunity, they have been developed a number of different treatment approaches to stimulate anti-tumour immune responses, such as anti-tumour vaccination, adoptive transfer of immune cells, inhibition of immune suppression and use of certain cytokines that activate innate immunity cells (Arosa, F.A. *et al.* 2012; Abbas, A.K. *et al.* 2014). Each of these treatments are designed to increase or restore immune function through different mechanisms of action. The mechanisms include 1), stimulation of anti-tumour response, either by increasing the number of effector cells or by producing one or more effector soluble mediators such as cytokines; 2) decreasing suppressor mechanisms; 3) modification of tumour cells to increase their immunogenicity and make them more susceptible to immunologic defense; and 4) improved tolerance to cytotoxic drugs or radiotherapy (Ampie, L. *et al.* 2015).

Immunotherapeutic approaches under clinical development for GBM can broadly be classified into cellular therapies, monoclonal antibody therapy, checkpoint blockade and vaccine therapy (Table 1) (Reardon, D.A. *et al.* 2014; Ampie, L. *et al.* 2015).

**Table 1 - Immunotherapy approaches for GBM patients**. (Adapted from: Reardon, D.A. et al. 2014)

| Immunotherapeutic Approach | Class of Agent | Representative Therapeutic |
|---|---|---|
| **Cellular** | Adoptive T-cell transfer | |
| | Chimeric antigen receptor-engineered T cell | CTL019 |
| **Vaccination** | Tumour-associated antigen vaccine | ICT-107; IMA950 |
| | Tumour-specific antigen vaccine | Rindopepimut (EGFRvIII) |
| | Whole tumour lysate vaccine | DCVax |
| | HSP96 vaccine | HSPPC-96 |
| | Tumour-specific mutation vaccine | |
| **Immunomodulation** | Anti-CTLA-4 Mab | Ipilimumab; tremelimumab |
| | Anti-PD-1 Mab | Nivolumab; labrolizumab; pidilizumab |
| | Anti-PD-L1 MAb | BMS-936559;MPDL3280A; MEDI4736 |
| | Treg-depleting reagent | Dacluzimab |

# AIMS OF THE STUDY

# II. AIMS OF THE STUDY

The main goal of this project was the evaluation of immunomodulatory capacity of extracellular products of glioblastoma cells, in vitro. Thus, our working hypothesis was that the products excreted by glioblastoma cells have the ability to modulate host immune response.

Therefore, and in order to confirm our hypothesis we divided our work in specific aims:

- Evaluate the effect of extracellular products of glioblastoma cell in T-cell activation;
- Measure the effects of extracellular products of glioblastoma cell in production of IL-10 by T cells;
- Evaluate the effect of extracellular products of glioblastoma cell on function and differentiation of macrophages.

# MATERIAL & METHODS

# III. MATERIAL & METHODS

## 1. Cell culture

Human glioblastoma astrocytoma cell line U251 was obtained from HPA Culture Collections and supplied by Sigma (Sigma product no. 09063001) kindly provided by Dra. Paula Soares. U251 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4,5 g/l) medium with Sodium Pyruvate, with Stable Glutamine (Capricorn Scientific GmbH), supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific GmbH), 25mM HEPES (Capricorn Scientific GmbH) and 1% (v/v) penicillin-streptomycin (PenStrep, Capricorn Scientific GmbH) at 37ºC in a humidified incubator with 5% $CO_2$.

Cells were maintained by replacement of fresh medium every three days. Cell lines were sub-cultured by trypsinization [Trypsin-EDTA (0.05 %) in DPBS (1x) Capricorn Scientific GmbH] when they reached 80% of confluence. Afterwards the cells were frozen in vials with freeze medium [complete growth medium supplemented with 10% (v/v) of dimethyl sulfoxide (DMSO, Merck Millipore)] in cell freezing containers, containing $1x10^6$ cells/ml.

## 2. Collection of extracellular products from glioblastoma cells culture

The extracellular products were obtained from cell culture of glioblastoma. A vial of cells was thawed and cultured in a $25cm^3$ flask with DMEM-HG medium supplemented with 20% FBS, 25mM HEPES and 1% PenStrep. After two days, the cells were trypsinized and sub-cultured in three $25cm^3$ flasks (corresponding to three different time-points). At each time point (24, 48 and 72 hours after trypsinization), the extracellular products were collected and were separated into high molecular weight (HMW) and low molecular weight (LMW) fractions using Macrosep® Advance Centrifugal Devices 10kDa (Pall Corporation). The HMW fraction was filtrated with syringe filters with 0,2 µm of pore size. Afterwards the extracellular products were aliquoted and frozen in liquid nitrogen for later use.

## 3. Assay of immunomodulatory capacity of extracellular products in PBMCs

Human mononuclear cells were isolated from peripheral blood of healthy volunteers (Centro Hospitalar do Porto, Oporto, Portugal) by Lymphoprep™ (STEMCELL Technologies) density centrifugation. Mononuclear cells were plated in 96-well conical bottom plates in RPMI medium supplemented with 10% FBS, 25mM HEPES and 1% PenStrep and were treated either with extracellular products of HMW (50% v/v), LMW (50%v/v) or with HMW+LMW (50% v/v) or with RPMI. Three hours later, concanavalin A from *Canavalia ensiformis* (Jack bean) (ConcA, 2,5 µg/mL, Sigma-Aldrich®), a mitogen that preferentially activates T-cells, was added to the plate and placed at 37ºC in a humidified incubator with 5% $CO_2$ overnight (16-24 hours). After incubation, supernatants were removed, centrifuged at 500g for 10 min and stored at -80ºC for further quantification of cytokines. T-cell activation was assessed by flow cytometry. As a positive control, PBMC from healthy donors were treated with culture medium (RPMI) and ConcA. PBMCs from healthy donor PBMCs treated only with culture medium (RPMI), was used as a negative control.

## 4. Flow Cytometry

PE anti-human CD3 (HIT3a; Biolegend), Biotin anti-human CD69 (FN50; Biolegend) and PE/Cy5 Streptavidin (Biolegend) antibodies were used for cell surface staining. Sub-analysis of the T-cell population activation was based on the gated surface expression of CD3 (T cell marker) and CD69 (early activation marker). Flow cytometry acquisition was done with BD FACSCanto™ II (BD Biosciences) and data analysis was with FlowJo software (TreeStar).

## 5. Isolation and differentiation of macrophages

Human mononuclear cells were isolated from peripheral blood of healthy volunteers (Centro Hospitalar do Porto, Oporto, Portugal) by Lymphoprep™ (STEMCELL Technologies) density centrifugation. Mononuclear cells were submitted to a second density gradient using Percoll™ (GE Healthcare) to separate monocytes from lymphocytes. Subsequently, the monocytes were plated in 96-well flat bottom plates in RPMI medium supplemented with 10% FBS, 25mM HEPES and 1% PenStrep. For macrophage stimulation 2 µg/mL of Recombinant Human Granulocyte Macrophage Colony Stimulating Factor (rh GM-CSF, ImmunoTools) was added at the first day of differentiation, and at the

third and sixth days was added RPMI medium supplemented with 10% FBS, 25mM HEPES and 1% PenStrep and 1 μg/mL of rh GM-CSF. At the seventh day the macrophages are differentiated.

## 6. Intracellular killing assay of macrophages modulated by extracellular products

Extracellular products (LMW or HMW fractions) were added with RPMI medium (50% v/v) supplemented with 10% FBS and 25mM HEPES (without PenStrep) to the differentiated macrophages ($7^{th}$ day). On the $11^{th}$ day, supernatants were removed for further quantification of cytokines and after 3 washes the macrophages were infected with $1{,}54x10^5$ CFU/well of bacteria (*Staphylococcus aureus*). Microplates were incubated for 3h at 37ºC in 5% $CO_2$ for bacterial phagocytosis. At different time-points (0h, 3h, 24h and 48h), culture supernatants of infected macrophages were removed by aspiration and cells were washed three times with RPMI medium supplemented with 20% FBS, 25mM HEPES and 1% PenStrep to remove extracellular bacteria. To quantify intracellular bacteria, the cells were lysed with saponin (0,1% final concentration). After the first time point, the infected macrophages, not treated with saponin, were further incubated in extracellular products of glioblastoma cell cultures. The colony-forming units (CFUs) of intracellular *S. aureus* were evaluated by plating serial dilutions of the supernatants in Todd-Hewitt agar medium.

## 7. Enzyme-linked immunosorbent assay (ELISA)

Detection of IL-10 in the supernatants of PBMCs cultures and detection of IL-10 and TNF-α in the supernatants of macrophages cultures was performed with an ELISA kit (ImmunoTools, GmbH) according to the manufacturer's instructions. Briefly, MaxiSorp™ high protein-binding plates (Nunc) were coated overnight at 4º C with the capture-antibody. Afterwards, the blocking-buffer was added to each well and incubate 1h at room temperature. After removing the blocking-buffer completely, the sample and the standard were added and incubated at room temperature for 2 h. Thereafter, the detector-antibody was transferred to each well and incubated 2 h at room temperature. Poly-HRP-Streptavidin-HS was added and after 30 minutes the TMB substrate was transferred to each well. When the enzymatic colour reaction was considered to be sufficient, the reaction was stopped by adding the stop solution. It should be noted that between each step the plate was washed 5x with washing-buffer.

# RESULTS

# IV. RESULTS

## 1. Extracellular products from glioblastoma cell culture inhibit T-Cell activation

T cells recognize pathogen fragments presented by APCs in the context of MHC molecules (Xing, Y. and Hogquist, K.A. 2012). Tumour microenvironment is characterized by the presence of immunosuppressive factors that can lead to the induction of T-cell tolerance or T-cell inactivation (Rabinovich, G.A. *et al.* 2007).

To determine if the extracellular products of glioblastoma cell culture could inhibit the activation of T-cells, PBMCs from healthy donors were activated with concanavalin A (ConcA), a mitogen that preferentially activates T-cells (Krauss, S. *et al.* 1999), in the presence or absence of extracellular products obtained from cell culture of glioblastoma. This extracellular products were collected at different time-points and separated into different fractions, HMW (high molecular weight) and LMW (low-molecular weight), in order to assess if the inhibitory activity is dependent on molecules with high or low molecular weight. T-cell activation was assessed by flow cytometry, using the surface marker CD3 to identify T cells and the early activation marker CD69 to assess T cell activation. As a positive control, PBMC from healthy donors were treated with culture medium (RPMI) and ConcA. PBMCs from healthy donor PBMCs treated only with culture medium (RPMI), was used as a negative control.

As depicted in figure 12 we could observe that after ConcA stimulation, the PBMCs from healthy donors treated with extracellular products of glioblastoma tumour cells expressed lower levels of CD69, when compared with PBMCs treated with ConcA alone. We also observe that the lower levels of $CD3^{+}CD69^{+}$ cells were recorded when both fractions (HMW and LMW) were added to PBMC cultures (figura 12B). As shown in figure 12C, the percentage of inhibition of T cells is elevated (> 50%) in the presence of both fractions (HMW and LMW) and remains practically unchanged for extracellular products collected at different time points.

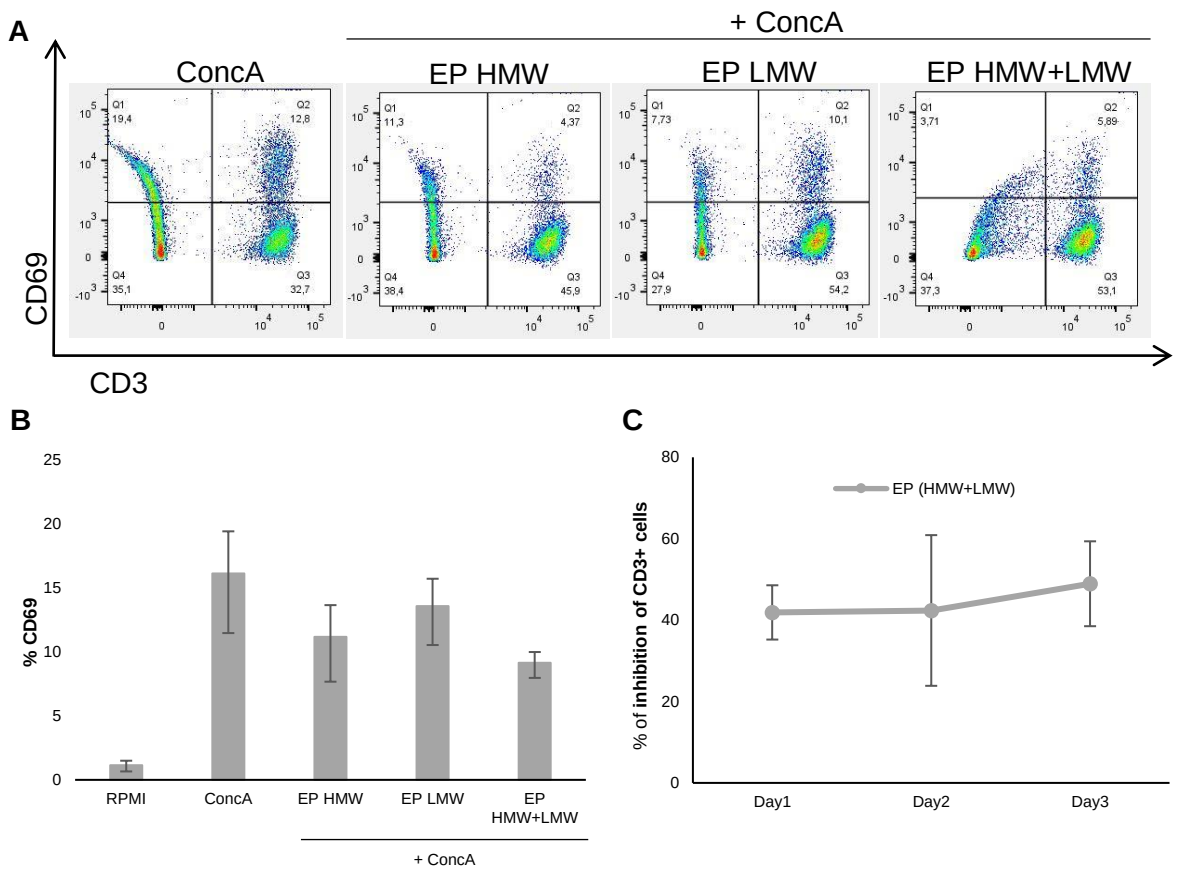


**Figure 12 - Extracellular products of glioblastoma cell culture reduces CD69 expression on T cells upon mitogenic stimulus.** PBMCs from healthy donors were treated either with HMW (50% v/v), LMW (50%v/v) or with HMW+LMW (50% v/v) or with RPMI (negative control) 3h before incubation with 2.5 µg/mL of ConcA or RPMI alone for 20h at 37ºC and 5% $CO_2$. After stimulation, lymphocyte activation was evaluated by flow cytometry analysis, using the early activation marker CD69 to assess cell activation. **A and B**, extracellular products of glioblastoma cell culture induce a decrease in the number of $CD3^+CD69^+$ compared with positive control. **A,** representative FACS plots are shown. **B,** the percentage of $CD69^+$ cells is lower in the presence of extracellular products of high and low molecular weight (EP HMW+LMW). **C,** the percentage of inhibition of T cells remains practically unchanged in the presence of EP HMW+LMW collected at different time-points**.** The percentage of inhibition of T cells was calculated relative to the measured levels ConcA. Data are the mean ± average deviation from two independent experiments.

## 2. Effects of extracellular products from glioblastoma cell culture on lymphocyte production of IL-10

IL-10 is an immunomodulatory cytokine that is frequently upregulated in various types of cancer (Sato, T. *et al.* 2011). IL-10 is produced by multiple cell types and has been shown to directly affect pro-inflammatory cytokine production, leukocyte activation and downregulates the expression of anti-microbial molecules on activated phagocytes (Moore, K.W. *et al.* 2001;Sato, T. *et al.* 2011).

To asses if the inhibitory activity of the extracellular products from glioblastoma cell cultures observed in Figure 12 was associated with IL-10 production, we quantified IL-10

levels in supernatants of PBMC stimulated with ConcA in the presence or absence of the extracellular products of glioblastoma cells.

The results show that higher levels of IL-10 production was observed in PBMCs treated with both fractions (HMW and LMW) as compared to lymphocytes treated with ConcA alone (figure 13). This was observed for extracellular products collected at different time-points and is in accordance to what was observed in Figure 12, where the maximum inhibitory activity was observed also when both fractions were added simultaneous to PBMCs. Interestingly, we also noted that extracellular products alone were not able to induce IL-10 production, rather inducing IL-10 production only if an mitogen stimulus was added.

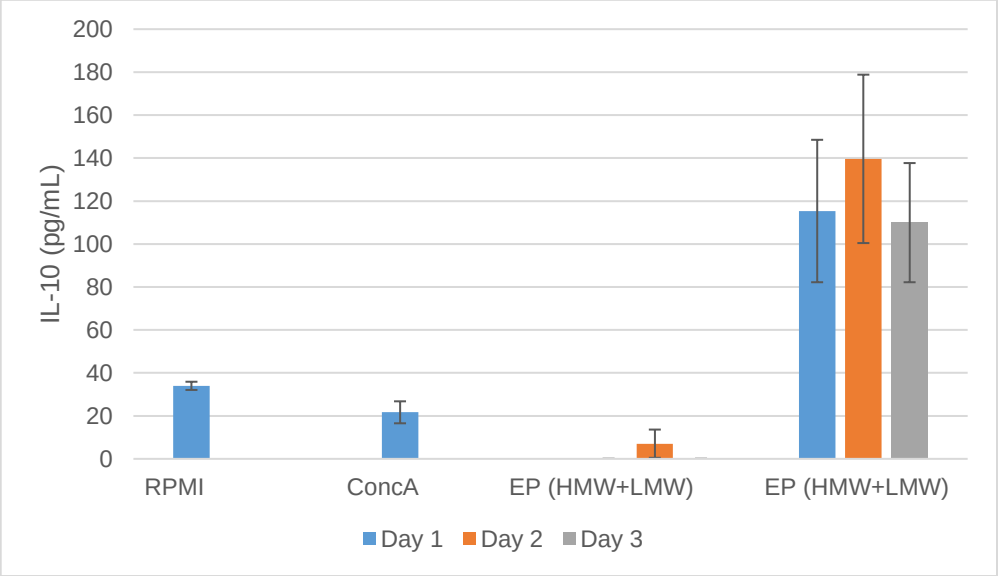


**Figure 13 - Quantification of IL-10 in supernatants of lymphocytes treated with extracellular products from glioblastoma cell culture.** Cytokine production was measured in unstimulated lymphocytes (RPMI), in stimulated lymphocytes (ConcA), in lymphocytes treated with the combination of extracellular products of high molecular weight and low molecular weight (EP HMW+LMW) and in lymphocytes treated with the combination of extracellular products of high molecular weight and low molecular weight (EP HMW+LMW) plus ConcA. Data are the mean ± average deviation from two independent experiments.

### 3. Effect of extracellular products from glioblastoma cell culture on function and differentiation of macrophages

To investigate the effect of extracellular products from glioblastoma cell culture on the ability of macrophages to kill intracellular bacteria, monocytes were isolated from the PBMCs of HDs and cultured for 7 d in the presence of GM-CSF. The differentiated macrophages were stimulated with the extracellular products of glioblastoma cells, with LMW or HMW fractions. After 3 days, *Staphylococcus aureus* was added to the macrophage culture in a multiplicity of infection (MOI) of 1.0. After 3h, the extracellular bacteria was removed by washing the wells three times with RPMI plus antibiotic. At the

indicated time-points saponin was added to the wells, in order to facilitate the destruction of plasma and phagosomal membranes of macrophages by interacting with cholesterols (Kaneko, M. *et al.* 2016), allowing the release of the surviving bacteria into the medium. The colony-forming units (CFUs) of intracellular *S. aureus* were evaluated by plating serial dilutions of the supernatants in Todd-Hewitt agar medium.

Results show an increase of intracellular bacteria in macrophages treated with extracellular products from glioblastoma cell culture of high molecular weight (EP HMW) at early time-points, namely 3h after the first incubation period (figure 14). When compared to the negative control (RPMI), macrophages treated with extracellular products of HMW or LMW have a lower ability to kill bacteria after phagocytosis.

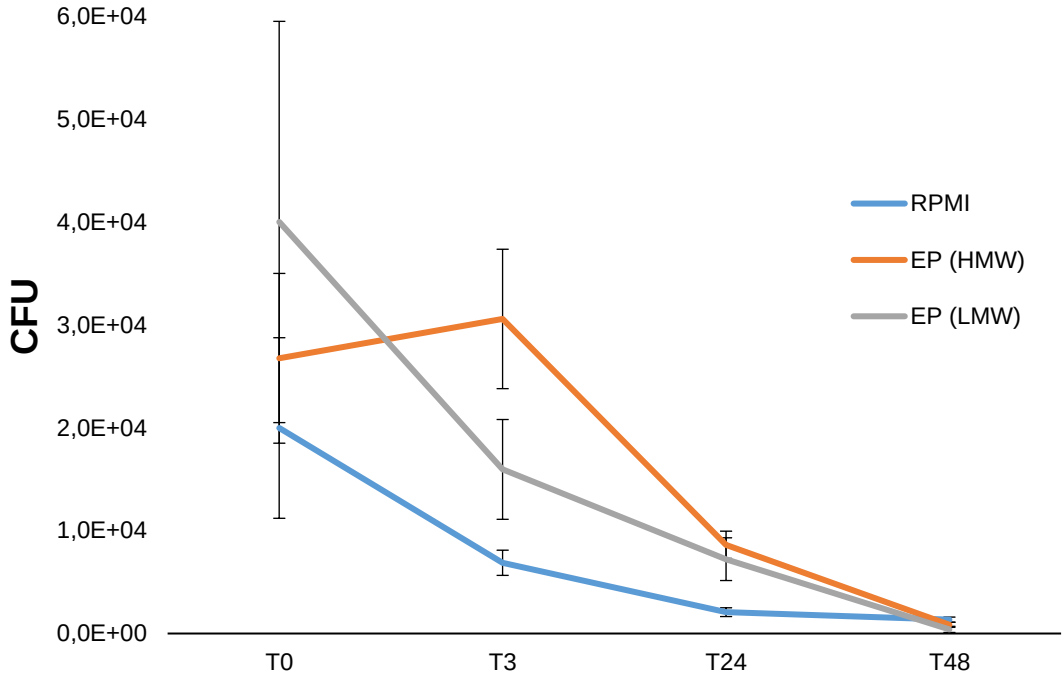


**Figure 14 - Extracellular products from glioblastoma cells reduce macrophage function**. Differentiated macrophages were treated with RPMI (negative control) or with extracellular products from glioblastoma cell culture with high molecular weight (PE HMW) or low molecular weight (EP LMW). $1.54x10^5$ CFU of *Staphylococcus aureus* was added to macrophages cultures and incubated for 3h. After incubation, extracellular bacteria was removed. At the indicated time points (0h, 3h, 24h and 48h) saponin was added to the wells and intracellular viable bacteria were counted on agar plates. Data are the mean ± average deviation from two independent experiments.

Due to the inability of macrophages to kill intracellular bacteria at time-point 'T3', it was investigated the effect of extracellular products of glioblastoma culture cells in polarization of macrophages into M1 or M2 phenotype. To this end, it was quantified the production of the IL-10 and TNF-α by ELISA in the supernatants of macrophage cultures.

The IL-10/TNF-α ratio is showed in figure 15. The IL-10 / TNF-α ratio is greater than 1 in macrophages conditioned with extracellular products of high molecular weight (EP HMW), which means that there is increased production of IL-10 compared to production of TNF-α by macrophages, indicating a differentiation in a M2 phenotype. This phenotype is associated with immunosuppression and contributes in vivo for tumour cell growth (Chanmee, T. *et al.* 2014) .

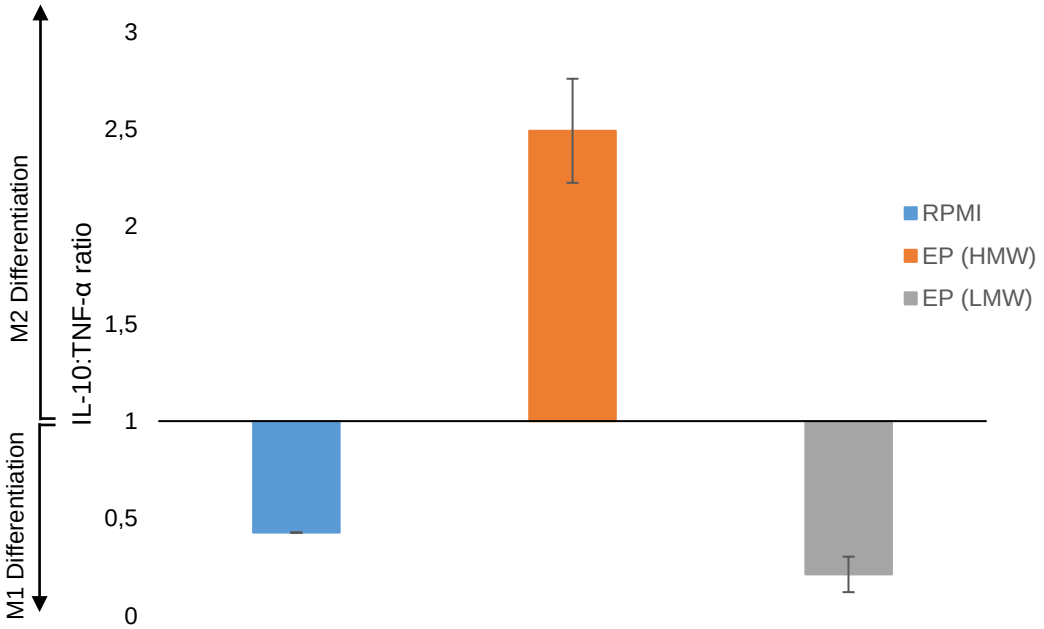


**Figure 15 - IL-10/ TNF-α ratio in supernatants of macrophages conditioned with extracellular products**. Before addition of the bacteria in the previous experiment, supernatants of macrophage cultures were collected. IL-10 and TNF-α present in supernatants were quantified by ELISA. Extracellular products with high molecular weight (PE HMW) induce an increase in production of IL-10 compared to production of TNF-α. Data are the mean ± average deviation from two independent experiments.

# DISCUSSION

# V. DISCUSSION

As referred initially, the main purpose of this work was to evaluate the immunomodulatory capacity of extracellular products of glioblastoma cells. The immunologic properties of extracellular products from glioblastoma cells have not been defined previously, and to our knowledge, this is the first study that suggests an immunomodulatory effect by extracellular factors produced by tumour cells.

To investigate the immune properties of extracellular products from glioblastoma cells, we used two different experimental approaches. In the first approach, the extracellular products from glioblastoma cells were used in immunological assays with PBMCs from healthy donors to determine the immunomodulatory effect of these extracellular products in the T cells. In the second approach, differentiated macrophages from monocytes isolated from the PBMCs of HDs were conditioned to extracellular products of glioblastoma cell cultures, in order to determine the existence of any immunomodulatory effect of extracellular products in macrophages, whether through loss of function or through differentiation into M2 phenotype.

T cells are the major players of the adaptive immune system and are able to recognize specific antigens presented by APC in the context of MHC molecules. However, in the tumour microenvironment T cells can undergo inactivation or tolerization leading to tumour escape and progression (Nagaraj, S. *et al.* 2007;Nurieva, R. *et al.* 2013;Abbas, A.K. *et al.* 2014). Several tumour-derived factors have been implicated in T cell suppression in glioblastoma patients. These include TGF-β2, PGE, IL-1, IL-10, IDO and FGL2 (Wrann, M. *et al.* 1987;Nduom, E.K. *et al.* 2015;Perng, P. and Lim, M. 2015).

In this work, it was possible to observe that the extracellular products of glioblastoma cells, lead to a decrease in T cell activation. In addition, was observed an increase of IL-10 production by T cells, which is more indicative of an immunosuppressive environment. IL-10 is an anti-inflammatory cytokine secreted by numerous cell types of the innate and adaptive immune system, which limits the excess of inflammation and prevents tissue damage (Dennis, K.L. *et al.* 2013). T cells, monocytes, macrophages, and DCs are particularly important both as targets and actors of IL-10-mediated immunosuppression (Moore, K.W. *et al.* 2001). Furthermore, IL-10 can also hinder cytotoxic T-lymphocyte effector functions by inducing and sustaining FOXP3 transcription factor expression in immunosuppressive Treg cells (Murai, M. *et al.* 2009). Cumulatively, the data presented herein could indicate that there is something secreted by the tumour, which contributes to immunosuppression, not only by inhibiting T cell activation, but also by the induction of

regulatory T cells in the tumour environment as a result of the increase in IL-10 production. Tregs, known as suppressor T cells, are able to suppress the proliferation of antigen-stimulated naïve T cells and other immune cells (B cells, NK cells, NKT cells, monocytes and DCs) (Schmidt, A. *et al.* 2012).

Macrophages, which are innate immune cells that play a broad role in host defense and the maintenance of tissue homeostasis, can be polarized into M1 or M2 phenotype depending on the microenvironment. Upon activation, M1 macrophages produce proinflammatory cytokines like TNF-α, IL-6, IL-12 and IL-23 (Chanmee, T. *et al.* 2014; Wang, N. *et al.* 2014). In contrast, M2 macrophages produce high levels of IL-10, have been considered to exert a tumour-promoting influence (Sica, A. *et al.* 2006; Hao, N.B. *et al.* 2012). Tumour-associated macrophages (TAMs) exhibit functions similar to those of M2 macrophages, can be activated in malignant tumours, and this may contribute to tumour metastasis, angiogenesis and immunosuppression (Mantovani, A. and Sica, A. 2010; Qian, B.Z. and Pollard, J.W. 2010).

In this report, we also showed that the extracellular products of high molecular weight of glioblastoma cells serves as a key regulator of macrophages differentiation. Our results indicate that there is something that actually is being secreted by glioblastoma cells, which leads to polarization to macrophages into M2 phenotype. This is supported by the increased of IL-10 production accompanied by a decrease of TNF-α production.

One of the main functions of macrophages is engulf the infectious agents, internalizing, and destroying them (Underhill, D.M. and Goodridge, H.S. 2012). Interestingly, we also observed that polarization of macrophage into M2 phenotype is associated with inhibition of specific function of macrophages, i.e., macrophages exhibit a decreased ability to kill intracellular bacteria after phagocytosis.

Different experimental approaches consistently showed that there is something excreted by tumour cells that exerts a direct immunomodulation on host immune cells, inducing IL-10 production by T cells and macrophages, decrease the T cell activation, and inducing the differentiation of monocytes into macrophages M2.

# CONCLUSION

# VI. CONCLUSION

The present study comprised the evaluation of the immunomodulatory capacity of extracellular products of glioblastoma cells. To this end, it evaluated the ability of extracellular products either to induce IL-10 production by T-cells or to inhibit T cell activation. It was also evaluated the effect of extracellular products in function and differentiation of macrophages.

Regardless of the experimental approaches, the data consistently demonstrated that there is something effectively secreted by tumour cells that exerts a direct immunomodulation on host immune cells, inducing IL-10 production by T cells and macrophages, decrease the T cell activation, and inducing the differentiation of monocytes into macrophages M2.

In summary, this study allows to infer an impact of extracellular products of glioblastoma cells in suppression of the immune response. Moreover, as it is something excreted can act from a distance, i.e., it is not something structural, blocking the immune response against tumour antigens, even being very specific. This fact could explain why immunotherapies directed against surface or structural antigens of tumours have failed.

# REFERENCES

# VII. REFERENCES


Abbas, A.K., Lichtman, A.H.H. and Pillai, S. (2014). Cellular and Molecular Immunology. Philadelphia, Elsevier Health Sciences.

Agrawal, N.S., Miller, R., Lal, R., Mahanti, H., Dixon-Mah, Y.N., DeCandio, M.L., Vandergrift, W.A., Varma, A.K., Patel, S.J., Banik, N.L., Lindhorst, S.M., Giglio, P. and Das, A. (2014). "Current Studies of Immunotherapy on Glioblastoma." J Neurol Neurosurg **1**(1).

Akira, S. (2011). "Innate immunity and adjuvants." Philos Trans R Soc Lond B Biol Sci **366**(1579): 2748-2755.

Alberts, B., Johnson, A., Lewis, J., Walter, P., Raff, M. and Roberts, K. (2002). Molecular Biology of the Cell 4th Edition. New York, Garland Science.

Alifieris, C. and Trafalis, D.T. (2015). "Glioblastoma multiforme: Pathogenesis and treatment." Pharmacol Ther **152**: 63-82.

Ampie, L., Woolf, E.C. and Dardis, C. (2015). "Immunotherapeutic Advancements for Glioblastoma." Front Oncol **5**.

Arosa, F.A., Cardoso, E.M. and Pacheco, F.C. (2012). Fundamentos de Imunologia. 2ª Edição. Lisboa, Lidel.

Avril, T., Vauleon, E., Tanguy-Royer, S., Mosser, J. and Quillien, V. (2011). "Mechanisms of immunomodulation in human glioblastoma." Immunotherapy **3**(4 Suppl): 42-44.

Balis, F.M. (1998). "The Goal of Cancer Treatment." Oncologist **3**(4): V.

Batista, F.D. and Harwood, N.E. (2009). "The who, how and where of antigen presentation to B cells." Nat Rev Immunol **9**(1): 15-27.

Boehm, T. and Bleul, C.C. (2007). "The evolutionary history of lymphoid organs." Nat Immunol **8**(2): 131-135.

Bonilla, F.A. and Oettgen, H.C. (2010). "Adaptive immunity." Journal of Allergy and Clinical Immunology **125**(2): S33-S40.

Bonneville, M., O'Brien, R.L. and Born, W.K. (2010). "Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity." Nat Rev Immunol **10**(7): 467-478.

Braza, M.S. and Klein, B. (2013). "Anti-tumour immunotherapy with Vγ9Vδ2 T lymphocytes: from the bench to the bedside." British Journal of Haematology **160**(2): 123-132.

Bush, N.A., Chang, S.M. and Berger, M.S. (2016). "Current and future strategies for treatment of glioma." Neurosurg Rev.

Caligiuri, M.A. (2008). "Human natural killer cells." Blood **112**(3): 461-469.

Chanmee, T., Ontong, P., Konno, K. and Itano, N. (2014). "Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment." Cancers (Basel) **6**(3): 1670-1690.

Chaplin, D.D. (2006). "1. Overview of the human immune response." J Allergy Clin Immunol **117**(2 Suppl Mini-Primer): S430-435.

Chaplin, D.D. (2010). "Overview of the immune response." J Allergy Clin Immunol **125**(2 Suppl 2): S3-23.

Cheng, M., Chen, Y., Xiao, W., Sun, R. and Tian, Z. (2013). "NK cell-based immunotherapy for malignant diseases." Cell Mol Immunol **10**(3): 230-252.

Chretien, A.S., Le Roy, A., Vey, N., Prebet, T., Blaise, D., Fauriat, C. and Olive, D. (2014). "Cancer-Induced Alterations of NK-Mediated Target Recognition: Current and Investigational Pharmacological Strategies Aiming at Restoring NK-Mediated Anti-Tumor Activity." Front Immunol **5**.

Cui, J., Chen, Y., Wang, H.Y. and Wang, R.F. (2014). "Mechanisms and pathways of innate immune activation and regulation in health and cancer." Hum Vaccin Immunother **10**(11): 3270-3285.

Dadi, S., Chhangawala, S., Whitlock, B.M., Franklin, R.A., Luo, C.T., Oh, S.A., Toure, A., Pritykin, Y., Huse, M., Leslie, C.S. and Li, M.O. (2016). "Cancer Immunosurveillance by Tissue-Resident Innate Lymphoid Cells and Innate-like T Cells." Cell **164**(3): 365-377.

Dasgupta, A. and Saxena, R. (2012). "Regulatory T cells: a review." Natl Med J India **25**(6): 341-351.

Dennis, K.L., Blatner, N.R., Gounari, F. and Khazaie, K. (2013). "Current status of IL-10 and regulatory T-cells in cancer." Curr Opin Oncol **25**(6): 637-645.

Dong, C. and Martinez, G.J. (2010). "T cells: the usual subsets." Nature reviews immunology.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. and Schreiber, R.D. (2002). "Cancer immunoediting: from immunosurveillance to tumor escape." Nat Immunol **3**(11): 991-998.

Dunn, G.P., Koebel, C.M. and Schreiber, R.D. (2006). "Interferons, immunity and cancer immunoediting." Nat Rev Immunol **6**(11): 836-848.

Dunn, G.P., Old, L.J. and Schreiber, R.D. (2004). "The three Es of cancer immunoediting." Annu Rev Immunol **22**: 329-360.

Eguizabal, C., Zenarruzabeitia, O., Monge, J., Santos, S., Vesga, M.A., Maruri, N., Arrieta, A., Riñón, M., Tamayo-Orbegozo, E., Amo, L., Larrucea, S. and Borrego, F. (2014). "Natural Killer Cells for Cancer Immunotherapy: Pluripotent Stem Cells-Derived NK Cells as an Immunotherapeutic Perspective." Front Immunol **5**.

Elsabahy, M. and Wooley, K.L. (2013). "Cytokines as biomarkers of nanoparticle immunotoxicity." Chem Soc Rev **42**(12): 5552-5576.

Ervik, M., Lam, F., Ferlay, J., Mery, L., Soerjomataram, I. and Bray, F. (2016). "Cancer Today." Lyon, France: International Agency for Research on Cancer. Cancer Today. Available from: http://gco.iarc.fr/today, accessed [30/08/2016].

Esteller, M., Gaidano, G., Goodman, S.N., Zagonel, V., Capello, D., Botto, B., Rossi, D., Gloghini, A., Vitolo, U., Carbone, A., Baylin, S.B. and Herman, J.G. (2002). "Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma." J Natl Cancer Inst **94**(1): 26-32.

Ezekowitz, R.A.B. and Hoffmann, J.A. (2002). Innate Immunity, Humana Press.

Fitzmaurice, C., Dicker, D., Pain, A., Hamavid, H., Moradi-Lakeh, M., MacIntyre, M.F., Allen, C., Hansen, G., Woodbrook, R., Wolfe, C., Hamadeh, R.R., Moore, A., Werdecker, A., Gessner, B.D., Te Ao, B., McMahon, B., Karimkhani, C., Yu, C., Cooke, G.S., Schwebel, D.C., Carpenter, D.O., Pereira, D.M., Nash, D., Kazi, D.S., De Leo, D., Plass, D., Ukwaja, K.N., Thurston, G.D., Yun Jin, K., Simard, E.P., Mills, E., Park, E.K., Catala-Lopez, F., deVeber, G., Gotay, C., Khan, G., Hosgood, H.D., 3rd, Santos, I.S., Leasher, J.L., Singh, J., Leigh, J., Jonas, J.B., Sanabria, J., Beardsley, J., Jacobsen, K.H., Takahashi, K.,

Franklin, R.C., Ronfani, L., Montico, M., Naldi, L., Tonelli, M., Geleijnse, J., Petzold, M., Shrime, M.G., Younis, M., Yonemoto, N., Breitborde, N., Yip, P., Pourmalek, F., Lotufo, P.A., Esteghamati, A., Hankey, G.J., Ali, R., Lunevicius, R., Malekzadeh, R., Dellavalle, R., Weintraub, R., Lucas, R., Hay, R., Rojas-Rueda, D., Westerman, R., Sepanlou, S.G., Nolte, S., Patten, S., Weichenthal, S., Abera, S.F., Fereshtehnejad, S.M., Shiue, I., Driscoll, T., Vasankari, T., Alsharif, U., Rahimi-Movaghar, V., Vlassov, V.V., Marcenes, W.S., Mekonnen, W., Melaku, Y.A., Yano, Y., Artaman, A., Campos, I., MacLachlan, J., Mueller, U., Kim, D., Trillini, M., Eshrati, B., Williams, H.C., Shibuya, K., Dandona, R., Murthy, K., Cowie, B., Amare, A.T., Antonio, C.A., Castaneda-Orjuela, C., van Gool, C.H., Violante, F., Oh, I.H., Deribe, K., Soreide, K., Knibbs, L., Kereselidze, M., Green, M., Cardenas, R., Roy, N., Tillmann, T., Li, Y., Krueger, H., Monasta, L., Dey, S., Sheikhbahaei, S., Hafezi-Nejad, N., Kumar, G.A., Sreeramareddy, C.T., Dandona, L., Wang, H., Vollset, S.E., Mokdad, A., Salomon, J.A., Lozano, R., Vos, T., Forouzanfar, M., Lopez, A., Murray, C. and Naghavi, M. (2015). "The Global Burden of Cancer 2013." JAMA Oncol **1**(4): 505-527.

Genin, M., Clement, F., Fattaccioli, A., Raes, M. and Michiels, C. (2015). "M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide." BMC Cancer **15**(1): 1-14.

Gnerlich, J.L., Mitchem, J.B., Weir, J.S., Sankpal, N.V., Kashiwagi, H., Belt, B.A., Porembka, M.R., Herndon, J.M., Eberlein, T.J., Goedegebuure, P. and Linehan, D.C. (2010). "Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer." J Immunol **185**(7): 4063-4071.

Gogoi, D. and Chiplunkar, S.V. (2013). "Targeting gamma delta T cells for cancer immunotherapy: bench to bedside." Indian J Med Res **138**(5): 755-761.

Golubovskaya, V. and Wu, L. (2016). "Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy." Cancers (Basel) **8**(3).

Gonzalez-Carmona, M.A., Marten, A., Hoffmann, P., Schneider, C., Sievers, E., Schmidt-Wolf, I.G., Sauerbruch, T. and Caselmann, W.H. (2006). "Patient-derived dendritic cells transduced with an a-fetoprotein-encoding adenovirus and co-cultured with autologous cytokine-induced lymphocytes induce a specific and strong immune response against hepatocellular carcinoma cells." Liver Int **26**(3): 369-379.

Gray, D. (2002). "A role for antigen in the maintenance of immunological memory." Nat Rev Immunol **2**(1): 60-65.

Gregersen, P.K. and Behrens, T.W. (2006). "Genetics of autoimmune diseases--disorders of immune homeostasis." Nat Rev Genet **7**(12): 917-928.

Guery, L. and Hugues, S. (2015). "Th17 Cell Plasticity and Functions in Cancer Immunity." Biomed Res Int **2015**: 314620.

Guo, J. and Zhou, X. (2015). "Regulatory T cells turn pathogenic." Cell Mol Immunol **12**(5): 525-532.

Hanahan, D. and Weinberg, R.A. (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hao, N.B., Lü, M.H., Fan, Y.H., Cao, Y.L., Zhang, Z.R. and Yang, S.M. (2012). "Macrophages in Tumor Microenvironments and the Progression of Tumors." Clin Dev Immunol **2012**.

Hou, L.C., Veeravagu, A., Hsu, A.R. and Tse, V.C. (2006). "Recurrent glioblastoma multiforme: a review of natural history and management options." Neurosurg Focus **20**(4): E5.

Kaneko, M., Emoto, Y. and Emoto, M. (2016). "A Simple, Reproducible, Inexpensive, Yet Old-Fashioned Method for Determining Phagocytic and Bactericidal Activities of Macrophages." Yonsei Med J **57**(2): 283-290.

Khan, F.A., Akhtar, S.S. and Sheikh, M.K. (2005). "Cancer Treatment - Objectives and Quality of Life Issues." Malays J Med Sci **12**(1): 3-5.

Kim, R., Emi, M. and Tanabe, K. (2007). "Cancer immunoediting from immune surveillance to immune escape." Immunology **121**(1): 1-14.

Kondo, M. (2010). "Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors." Immunol Rev **238**(1): 37-46.

Krauss, S., Buttgereit, F. and Brand, M.D. (1999). "Effects of the mitogen concanavalin A on pathways of thymocyte energy metabolism." Biochim Biophys Acta **1412**(2): 129-138.

Kumar, V., Abbas, A.K. and Aster, J.C. (2014). Robbins & Cotran Pathologic Basis of Disease, Elsevier Health Sciences.

Lam, S.S., Zhou, F., Hode, T., Nordquist, R.E., Alleruzzo, L., Raker, J. and Chen, W.R. (2015). "Advances in strategies and methodologies in cancer immunotherapy." Discov Med **19**(105): 293-301.

Li, B. and Wang, R.-F. (2012). Mechanisms and Control of Regulatory T Cells in Cancer. Innate Immune Regulation and Cancer Immunotherapy. Wang, R. New York, NY, Springer New York**:** 195-216.

Li, Z., Li, D., Tsun, A. and Li, B. (2015). "FOXP3+ regulatory T cells and their functional regulation." Cell Mol Immunol **12**(5): 558-565.

Linehan, E. (2015). "Ageing and the immune system: focus on macrophages." **5**(1): 14-24.

Llaguno-Munive, M., Medina, L.A., Jurado, R., Romero-Piña, M. and Garcia-Lopez, P. (2013). "Mifepristone improves chemo-radiation response in glioblastoma xenografts." Cancer Cell Int **13**: 29.

Loeb, L.A. and Harris, C.C. (2008). "Advances in chemical carcinogenesis: a historical review and prospective." Cancer Res **68**(17): 6863-6872.

Ma, C.S. and Deenick, E.K. (2014). "Human T follicular helper (Tfh) cells and disease." Immunol Cell Biol **92**(1): 64-71.

Ma, Y., Shurin, G.V., Peiyuan, Z. and Shurin, M.R. (2013). "Dendritic Cells in the Cancer Microenvironment." J Cancer **4**(1): 36-44.

Madureira, P., de Mello, R.A., de Vasconcelos, A. and Zhang, Y. (2015). "Immunotherapy for lung cancer: for whom the bell tolls?" Tumour Biol **36**(3): 1411-1422.

Mantia-Smaldone, G.M. and Chu, C.S. (2013). "A review of dendritic cell therapy for cancer: progress and challenges." BioDrugs **27**(5): 453-468.

Mantovani, A. and Sica, A. (2010). "Macrophages, innate immunity and cancer: balance, tolerance, and diversity." Curr Opin Immunol **22**(2): 231-237.

Martinez, F.O. and Gordon, S. (2014). "The M1 and M2 paradigm of macrophage activation: time for reassessment." F1000Prime Rep **6**.

Mills, K.H.G. (2004). "Regulatory T cells: friend or foe in immunity to infection?" Nat Rev Immunol **4**(11): 841-855.

Mittal, D., Gubin, M.M., Schreiber, R.D. and Smyth, M.J. (2014). "New insights into cancer immunoediting and its three component phases — elimination, equilibrium and escape." Curr Opin Immunol **27**: 16-25.

Mogensen, T.H. (2009). "Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses." Clin Microbiol Rev **22**(2): 240-273.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L. and O'Garra, A. (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C.L., Cheroutre, H. and Kronenberg, M. (2009). "Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis." Nat Immunol **10**(11): 1178-1184.

Murphy, K.M. (2011). Janeway's Immunobiology, 8th edition. New York, Garland Science, Taylor & Francis Group.

Murray, P.J. and Wynn, T.A. (2011). "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol **11**(11): 723-737.

Nagaraj, S., Gupta, K., Pisarev, V., Kinarsky, L., Sherman, S., Kang, L., Herber, D., Schneck, J. and Gabrilovich, D.I. (2007). "Altered recognition of antigen is a novel mechanism of CD8(+) T cell tolerance in cancer." Nat Med **13**(7): 828-835.

Nduom, E.K., Weller, M. and Heimberger, A.B. (2015). "Immunosuppressive mechanisms in glioblastoma." Neuro Oncol **17 Suppl 7**: vii9-vii14.

Nothelfer, K., Sansonetti, P.J. and Phalipon, A. (2015). "Pathogen manipulation of B cells: the best defence is a good offence." Nat Rev Microbiol **13**(3): 173-184.

Nurieva, R., Wang, J. and Sahoo, A. (2013). "T-cell tolerance in cancer." Immunotherapy **5**(5): 513-531.

Ohkura, N., Kitagawa, Y. and Sakaguchi, S. (2013). "Development and maintenance of regulatory T cells." Immunity **38**(3): 414-423.

Okada, M., Hibino, S., Someya, K. and Yoshmura, A. (2014). "Regulation of regulatory T cells: epigenetics and plasticity." Adv Immunol **124**: 249-273.

Ostrom, Q.T., Bauchet, L., Davis, F.G., Deltour, I., Fisher, J.L., Langer, C.E., Pekmezci, M., Schwartzbaum, J.A., Turner, M.C., Walsh, K.M., Wrensch, M.R. and Barnholtz-Sloan, J.S. (2014). "The epidemiology of glioma in adults: a "state of the science" review." Neuro Oncol **16**(7): 896-913.

Ostrom, Q.T., Gittleman, H., Stetson, L., Virk, S.M. and Barnholtz-Sloan, J.S. (2015). "Epidemiology of gliomas." Cancer Treat Res **163**: 1-14.

Palucka, K. and Banchereau, J. (2012). "Cancer immunotherapy via dendritic cells." Nat Rev Cancer **12**(4): 265-277.

Pardee, A.D., Shi, J. and Butterfield, L.H. (2014). "Tumor-derived alpha-fetoprotein impairs the differentiation and T cell stimulatory activity of human dendritic cells." J Immunol **193**(11): 5723-5732.

Parkin, J. and Cohen, B. (2001). "An overview of the immune system." Lancet **357**(9270): 1777-1789.

Perng, P. and Lim, M. (2015). "Immunosuppressive Mechanisms of Malignant Gliomas: Parallels at Non-CNS Sites." Front Oncol **5**.

Qian, B.Z. and Pollard, J.W. (2010). "Macrophage diversity enhances tumor progression and metastasis." Cell **141**(1): 39-51.

Quail, D. and Joyce, J. (2013). "Microenvironmental regulation of tumor progression and metastasis." Nat Med **19**(11): 1423-1437.

Rabinovich, G.A., Gabrilovich, D. and Sotomayor, E.M. (2007). "IMMUNOSUPPRESSIVE STRATEGIES THAT ARE MEDIATED BY TUMOR CELLS." Annu Rev Immunol **25**: 267-296.

Raval, R.R., Sharabi, A.B., Walker, A.J., Drake, C.G. and Sharma, P. (2014). "Tumor immunology and cancer immunotherapy: summary of the 2013 SITC primer." J Immunother Cancer **2**: 14.

Reardon, D.A., Freeman, G., Wu, C., Chiocca, E.A., Wucherpfennig, K.W., Wen, P.Y., Fritsch, E.F., Curry, W.T., Jr., Sampson, J.H. and Dranoff, G. (2014). "Immunotherapy advances for glioblastoma." Neuro Oncol **16**(11): 1441-1458.

Sakaguchi, S., Vignali, D.A., Rudensky, A.Y., Niec, R.E. and Waldmann, H. (2013). "The plasticity and stability of regulatory T cells." Nat Rev Immunol **13**(6): 461-467.

Sato, T., Terai, M., Tamura, Y., Alexeev, V., Mastrangelo, M.J. and Selvan, S.R. (2011). "Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy." Immunol Res **51**(2-3): 170-182.

Schmidt, A., Oberle, N. and Krammer, P.H. (2012). "Molecular Mechanisms of Treg-Mediated T Cell Suppression." Front Immunol **3**.

Schwartz, R.H. (2012). "Historical overview of immunological tolerance." Cold Spring Harb Perspect Biol **4**(4): a006908.

Schwartzbaum, J.A., Fisher, J.L., Aldape, K.D. and Wrensch, M. (2006). "Epidemiology and molecular pathology of glioma." Nat Clin Pract Neurol **2**(9): 494-503; quiz 491 p following 516.

Shang, B., Liu, Y., Jiang, S.J. and Liu, Y. (2015). "Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis." Sci Rep **5**: 15179.

Sica, A., Schioppa, T., Mantovani, A. and Allavena, P. (2006). "Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy." Eur J Cancer **42**.

Silva-Santos, B., Serre, K. and Norell, H. (2015). "[gamma][delta] T cells in cancer." Nat Rev Immunol **15**(11): 683-691.

Steevels, T.A. and Meyaard, L. (2011). "Immune inhibitory receptors: essential regulators of phagocyte function." Eur J Immunol **41**(3): 575-587.

Swann, J.B. and Smyth, M.J. (2007). "Immune surveillance of tumors." J Clin Invest **117**(5): 1137-1146.

Teng, M.W., Galon, J., Fridman, W.H. and Smyth, M.J. (2015). "From mice to humans: developments in cancer immunoediting." J Clin Invest **125**(9): 3338-3346.

Thakkar, J.P., Dolecek, T.A., Horbinski, C., Ostrom, Q.T., Lightner, D.D., Barnholtz-Sloan, J.S. and Villano, J.L. (2014). "Epidemiologic and molecular prognostic review of glioblastoma." Cancer Epidemiol Biomarkers Prev **23**(10): 1985-1996.

Thomas, A.A., Ernstoff, M.S. and Fadul, C.E. (2012). "Immunotherapy for the Treatment of Glioblastoma." Cancer J **18**(1): 59-68.

Thompson, M.R., Kaminski, J.J., Kurt-Jones, E.A. and Fitzgerald, K.A. (2011). "Pattern Recognition Receptors and the Innate Immune Response to Viral Infection." Viruses **3**(6): 920-940.

Tripathi, S.K. and Lahesmaa, R. (2014). "Transcriptional and epigenetic regulation of T-helper lineage." Immunol Rev **261**(1): 62-83.

Turvey, S.E. and Broide, D.H. (2010). "Chapter 2: Innate Immunity." J Allergy Clin Immunol **125**(2 Suppl 2): S24-32.

Underhill, D.M. and Goodridge, H.S. (2012). "Information processing during phagocytosis." Nat Rev Immunol **12**(7): 492-502.

Waldhauer, I. and Steinle, A. (2008). "NK cells and cancer immunosurveillance." Oncogene **27**(45): 5932-5943.

Wang, N., Liang, H. and Zen, K. (2014). "Molecular Mechanisms That Influence the Macrophage M1–M2 Polarization Balance." Front Immunol **5**.

Warrington, R., Watson, W., Kim, H.L. and Antonetti, F.R. (2011). "An introduction to immunology and immunopathology." Allergy, Asthma & Clinical Immunology **7**(1): 1-8.

Wrann, M., Bodmer, S., de Martin, R., Siepl, C., Hofer-Warbinek, R., Frei, K., Hofer, E. and Fontana, A. (1987). "T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-beta." Embo j **6**(6): 1633-1636.

Xing, Y. and Hogquist, K.A. (2012). "T-cell tolerance: central and peripheral." Cold Spring Harb Perspect Biol **4**(6).

Yang, J., Zhang, L., Yu, C., Yang, X.F. and Wang, H. (2014). "Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases." Biomark Res **2**: 1.

Ye, J., Livergood, R.S. and Peng, G. (2013). "The Role and Regulation of Human Th17 Cells in Tumor Immunity." The American Journal of Pathology **182**(1): 10-20.